THE JOURNAL

OF

PHARMACOLOGY

AND

EXPERIMENTAL THERAPEUTICS

CONTENTS

Number 3, November, 1947

Number 4, December, 1947

CARDIOVASCULAR AND RESPIRATORY EFFECTS OF THE ANTI-HISTAMINE AGENT, β-DIMETHYLAMINOETHYL BENZHYDRYL ETHER HYDROCHLORIDE (BENADRYL HYDROCHLORIDE[1])

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While Loew et al. (1, 2, 3) were establishing pharmacotherapeutic actions of Rieveschl and Huber's (4) benzhydryl alkamine ethers, and Gruhzit and Fisken (5) were studying over-all toxicity, certain general pharmacodynamic studies were done to aid in selection of a promising antihistamine compound. The present purpose is to report results of some of this early work on the anesthetized dog and isolated rabbit heart, with particular reference to Benadryl Hydrochloride (BH).

METHODS. Dogs anesthetized with sodium phenobarbital were used in the study of intravenous effects. In several experiments the carotid sinus and vagus nerve trunks were prepared for rapid section during the experiment, and/or arrangements were made for recording crude electrocardiograms by means of a Junior Garceau[3] Electroencephalograph fitted with a special voltage adaptor. Heart rates were frequently determined from the manometer or ECG record.

Rabbit hearts were perfused by Langendorff's method with oxygenated Locke solution at constant pressure (38 cm.) and temperature (36–37°C.). Aortic valvular leakage and the small Thebesian return to the left heart were drained away through a small cannula inserted through the left ventricular wall. The major coronary outflow was collected in a graduate for measurement over one minute intervals each 1.5 min., or recorded electrically (6). Ventricular contractions were recorded. Injections were made into a rubber-tubing connecting the cannula with the system.

RESULTS. I. *Intravenous effects on arterial pressure* are illustrated in figures 1, 2A and B, and 3A. A primary fall (lasting until 25 sec. to >1.4 hr., median 2.0 min., after beginning of injection) was typically followed by a relatively long-lasting (7.3 min. to >2 hr., median >46 min. after beginning of injection) but small rise above the preinjection level. Similar general observations have been mentioned in connection with other subdivisions of the work on BH (3, 5). Particularly at the higher dose levels, the primary depressor phase persisted for as long as 1.4 hr. or more.

The rapidity with which the primary effect was reached (reciprocal of time to fall) was best correlated with rapidity of injection—mgm./kgm./min. (correlation coefficient, 0.41; P = 0.01 to 0.05). The scattergram of this relation revealed a minimal latent period of 7.5 to 8.3 sec. occurring occasionally over the entire range of injection rates. At increasing injection rates the longest latent periods apt to occur receded toward this minimal value. The minimal

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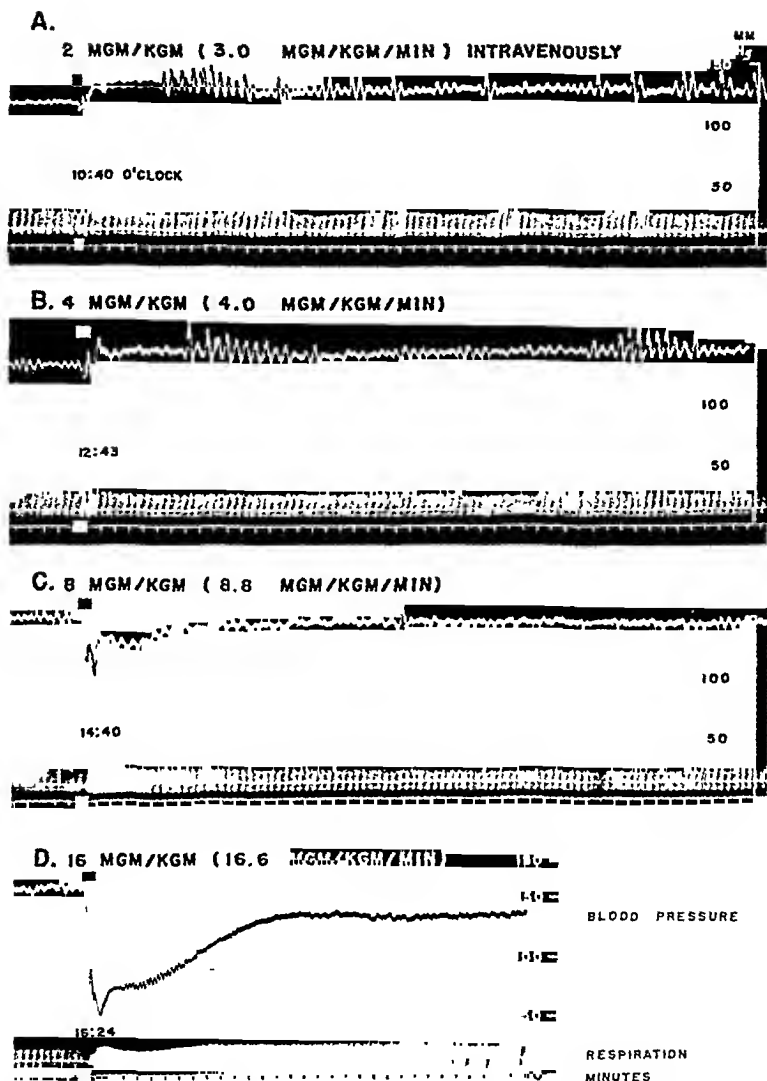
FIG. 1. Dog, ♀, 10.7 kgm. At 8:05, 160 mgm./kgm. sodium phenobarbital intraperitoneally. In all such experiments, respiration was recorded by a modification of (7).

and median (12 sec.) latencies suggest a site of action in the systemic or coronary vascular bed. The same order of latency was observed for chemoreflex hyperpneas (8, 9).
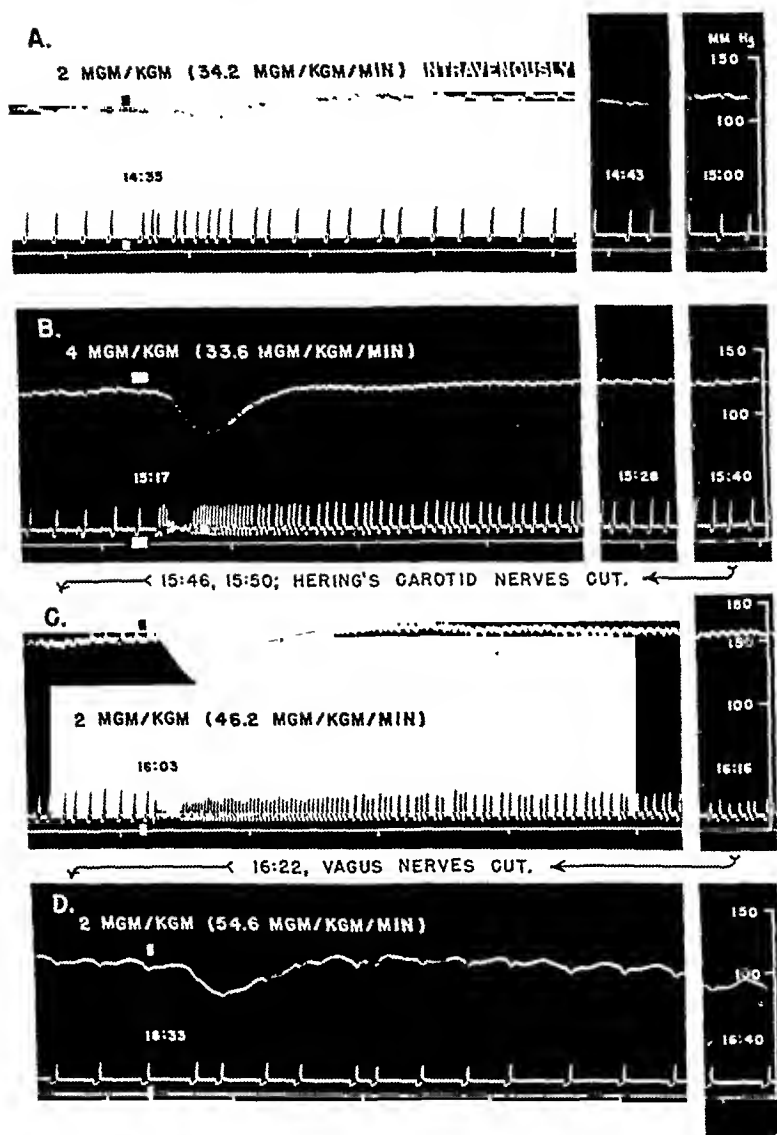
FIG. 2 Dog, ♀. 8 4 kgm 160 mgm /kgm. sodium phenobarbital intraperitoneally. Vagus and Hering's nerves prepared.

That both phases of pressure change are independent (a) of the important arterial reflex zones, (b) of vagal cardiac innervation, (c) of heart rate changes, (d) of peripheral cholinergic mechanisms, and (e) of nervous or mechanical re-
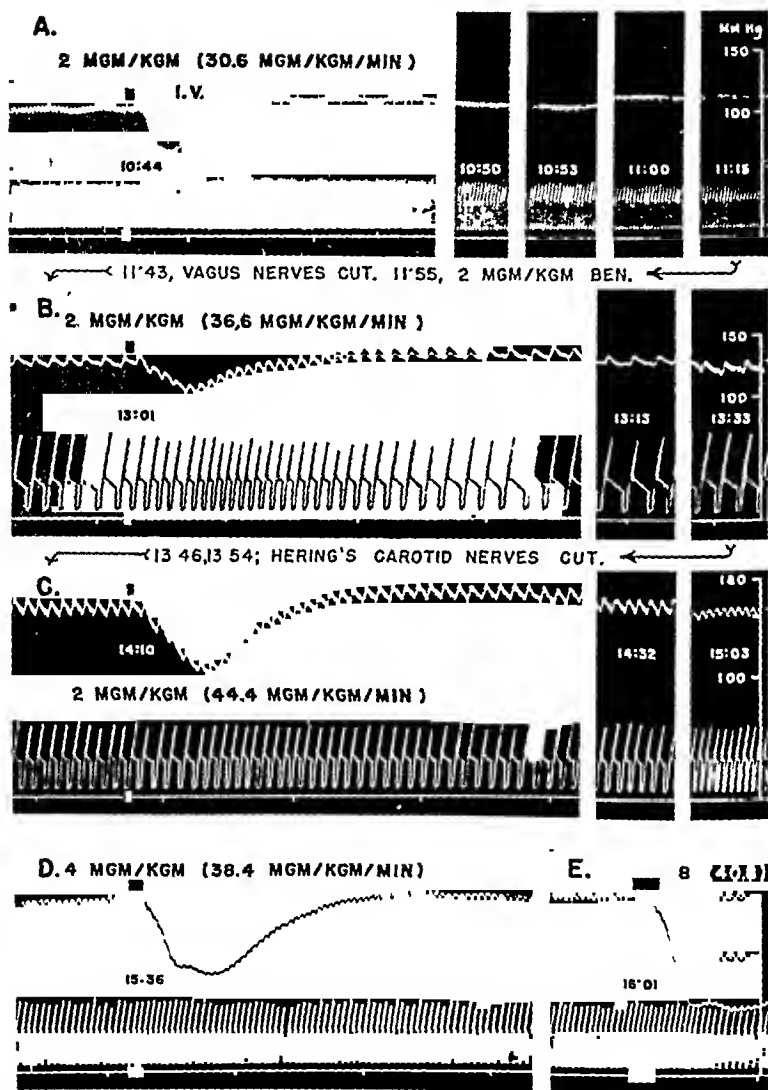
Fig 3. Dog, ♂, 12 0 kgm   At 8:15, 160 mgm /kgm sodium phenobarbital intraperitoneally   Vagus and Hering's nerves prepared

spiratory changes, was shown by their essential integrity (*a* and *b*) in the absence of the carotid and/or vagus nerves (Fig 2 and 3), (*c*) in the absence of significant heart rate changes, (*d*) in fully atropinized animals (1 mgm /kgm or more

of atropine sulfate; failure of depressor response to 10 μgm./kgm. of acetylcholine bromide), (e) in the absence of significant alteration in spontaneous respiration, and during constant artificial over-ventilation. The diphasic response to the drug is therefore essentially attributable to systemic vascular, or *inotropic* myocardial changes, or both, due to sites of action either local (but not cholinergic
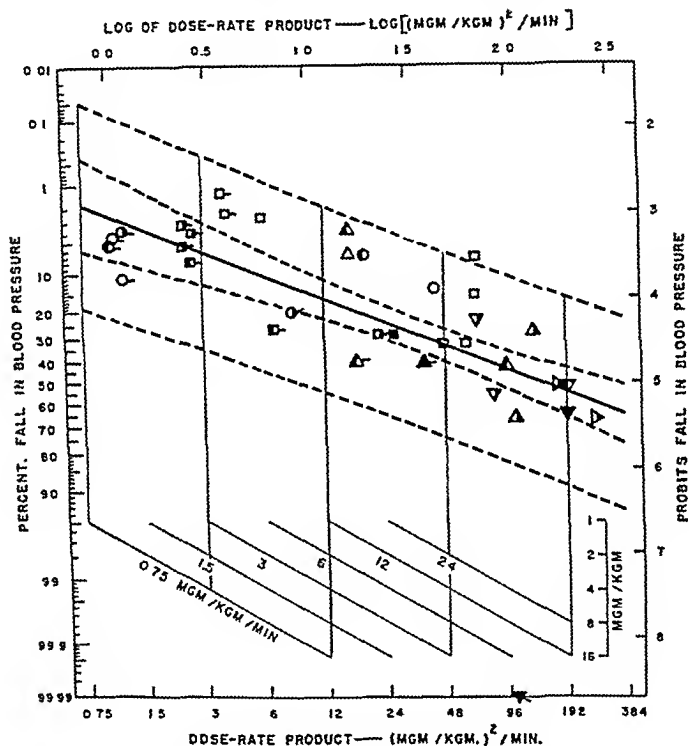
FIG. 4 RELATIONSHIP OF DOSE AND ITS RATE OF INJECTION TO AMOUNT OF PRIMARY FALL IN ARTERIAL BLOOD PRESSURE

See text Circles represent 1 mgm /kgm ; squares, 2, triangles, base down, 4, triangles, base up, 8, triangles, base to left, 16 Shading indicates preceding treatment in (mgm / kgm)²/min no shading, no previous injection, left half shaded, up to 0 1 ; right half shaded, > 0 1 to 1 0; total shaded, > 1 0 to 10 The tail indicates 170–176 mgm /kgm of phenobarbital, otherwise, 160–165

or anti-cholinergic) or at sympathetic ganglia or centers, or both. The occurrence in the isolated heart (*infra*) of a direct, transient, coronary dilatation followed by more persistent constriction suggests analogous direct actions on the systemic circulation. The occurrence in the isolated heart (*infra*) of a reversible negative inotropic action admits such a direct myocardial action as a possible factor in the depressor phase

Various measures of the primary blood-pressure fall (intact animals) were

plotted against various aspects of dosage. "Probits"[4] fall against log [(mgm./kgm.)^2/min.][5] yielded the scattergram (Fig. 4) that was by far most satisfactory as regards linearity of regression, average scatter relative to regression slope, and uniformity of scatter along the regression.
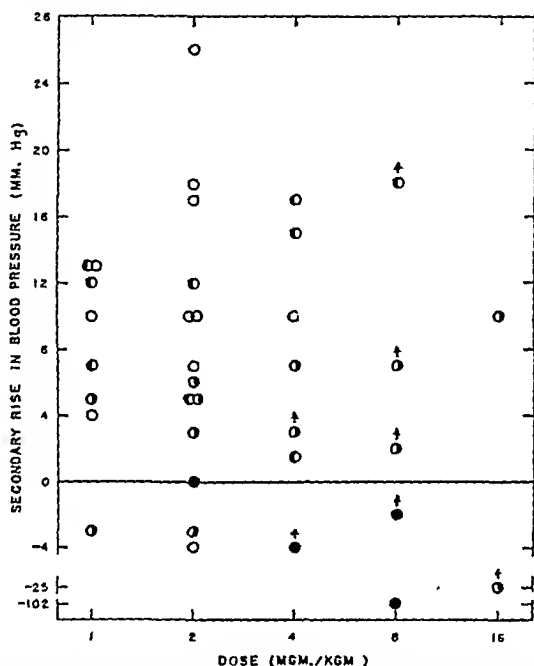
FIG 5. RELATIONSHIP OF DOSE TO MAXIMUM SECONDARY RISE IN ARTERIAL BLOOD PRESSURE, OR MINIMUM DEFICIT IN RECOVERY FROM PRIMARY FALL
Arrows indicate maximum not yet attained   Shading, as for fig 4, refers to preceding treatment

As illustrated in figure 5, there is no clear correlation of the amount of secondary rise with any aspect of the dose.   While in the composite scattergram there is no obvious influence of previous treatment, inspection of sequential results in individual animals reveals evidence of diminishing pressor phase as the drug or its effect must have accumulated in the organism from preceding injections.

II. *Effects on heart rate.*   One to 4 mgm./kgm. BH injected at varying rates resulted in variable changes in heart rate not exceeding 20%   In over half the cases there was a small acceleration, sometimes unmistakably associated with the

[4] "Probits" fall are derived by conversion of mm. Hg fall to percentage fall, and by reference to suitable tables (e g , (10) (11)), conversion of percentage effect to probit effect.   See also "Discussion".

[5] (Mgm /kgm)^2/min represents the product of dose (mgm /kgm.) by its rate of injection (mgm./kgm /min).   See also "Discussion"

injection, and lasting from a half hour to the duration of the recording. The greatest acceleration occurred during the drop in blood pressure, hence probably benefitted from pressoreceptive reflexes. However, small accelerations have persisted in spite of appearance of the pressor phase, or have been resumed following subsidence of the peak of this phase. They are therefore basically independent of arterial pressure. In other cases at these dose levels there were small decreases in heart rate, sometimes initiated (with reflex assistance) during the phase of rising pressure, sometimes preceded by a brief (partly reflex) acceleration during the drop in pressure, but occasionally beginning in association with the initial fall in pressure to be augmented (reflexly) during the subsequent rise. This latter, early deceleration occasionally preceded the acceleration when present.

Eight and 16 mgm./kgm. consistently caused the primary deceleration. It was usually well-sustained, but amounted to only 8 to 22% of the preinjection rates even at these high dose levels.

In none of the experiments in which Hering's carotid nerves and/or the vago-aortic nerve trunks were severed was the primary acceleration present in workable degree before cutting the nerves. This would not be surprising if the acceleration, when present, reflected an anti-cholinergic action dependent on vagal tone, because, as demonstrated by lack of signifiant change in basal heart rate after vagotomy, vagal tone was severely reduced (phenobarbital anesthesia). The small primary deceleration with 4 to 8 mgm./kgm. persisted after the major arterial presso- and chemoreceptive denervation and efferent vagal cardiac denervation. It probably represents a direct action of BH and/or a central or ganglionic sympathetic depression.

III. *Effects on the electrocardiogram.* Fine aspects of the recordings employed were not amenable to interpretation. No gross change in rhythm primarily attributable to BH was noted at any dose level. So far as it goes, this result agrees with that in man (12).

IV. *Effects on respiration.* Respiration was sometimes not disturbed by 1 mgm./kgm. injected slowly. More often this dose, at rates of 1.2 to 42.6 mgm./kgm. min., caused very slight to moderate stimulation lasting only a fraction to 2 or 3 minutes. With increasing doses and rates of injection the stimulation became intense, often lasting with gradually diminishing intensity for well over an hour with 8 or 16 mgm./kgm. (Fig. 1, 2A and B, 3A). In a majority of cases the first evidence of stimulation occurred shortly after the beginning of the fall in blood pressure. That a portion of the transient stimulations was due to pressoreceptive reflexes is likely (13, 14); e.g. Fig. 1A. However, many of them began simultaneously with or before the fall in pressure (e.g., Fig. 2A, B) and could not be entirely so explained. Furthermore, intense and prolonged stimulations outlasted the primary pressure fall and persisted in spite of the secondary rise in pressure.

Except in several of the slight responses, very early in the course of stimulation a restriction in depth of breathing began. It was present in all cases at 2 to 16 mgm./kgm. where the rate of injection exceeded 4.2 mgm. kgm./min. (e.g., Fig.

1C, D; 2A, B; 3A). In degree, it ranged from very slight (e.g., Fig. 2A) to complete apnea (e.g., Fig. 1D) of 10 to 30 seconds (one case of indefinite duration resulting in eventual cardiovascular failure and death). Duration of subnormal depth, roughly paralleling its intensity, ranged from a fraction of a minute to well over 2 hours. In cases of durable depth restriction the stimulation was expressed as moderate to intense acceleration (e.g., Fig. 1D) or shallow polypnea. In some cases with large doses accessory respiratory movements were observed to accompany the polypnea.

The general observation of respiratory stimulation and depth-restriction or apnea were mentioned in connection with other subdivisions of the work on BH (3, 5).

The depth-restricting action of BH and the major part of the acceleration were eliminated by vagotomy (e.g., Fig. 3B vs. A; 2D vs. C). That carotid denervation alone failed in this respect (e.g., Fig. 2C vs. B) probably places the responsibility for the depth restriction and major acceleration on vagal components other than aortic fibers, which are functionally homologous with the carotid system.

The anti-cholinergic action of BH (3, 15), its probable (myotropic) peripheral bronchiodilating action (16), and the apparent absence of concomitant parasympathetic actions all discount the likelihood of bronchioconstriction. An unusual central chemical augmentation of pulmonary proprioceptive respiratory control (cf. (17)) seems likely.

Carotid denervation removed a component of stimulation, expressed usually in depth, when the vagus nerves were still intact. After preliminary vagotomy (e.g., Fig. 3B) this simple mild residual stimulation was unmasked, to be essentially eliminated by subsequent carotid denervation. With vagus and carotid nerves all severed, even 4 or 8 mgm./kgm. of BH were essentially without effect on respiration (Fig. 2D; 3C, D, E).

That extra-aortic vagal fibers could subserve the simple stimulation of both rate and depth of respiration, pointed out above to be largely independent of blood-pressure changes, is not in accord with known physiology of respiratory control. The alternative is that the element of stimulation dependent on carotid (and presumably aortic) innervation is partly independent of blood pressure, i.e. partly chemoreceptive. Therefore, there is probably an interaction of chemoreceptive, pressoreceptive, and centrally altered pulmonary proprioceptive mechanisms involved in the respiratory effects of BH.

V. *Effects on the isolated heart.* The hydrochlorides of 18 benzhydryl alkamine ethers, including BH, have been studied by injection into the perfusion inlet to the heart. Typical of the compounds, in 50 μgm. dosage, was a slight to moderate decrease in amplitude of systolic contraction such as illustrated in figure 6. Under suitable experimental conditions these myocardial depressions were gradually recovered from, so that at the end of a 3 hr. experiment involving repeated injections the ventricular vigor might be as great or greater than initially (e.g., Fig. 6). Papaverine hydrochloride at this dose typically caused slight initial decrease followed by slightly supra-normal ventricular vigor.

In at least 14 of the 18 compounds, including BH, there was a qualitatively

uniform pattern of coronary flow change following the 50 μgm. dose.   An initial, very transient increase was followed by a more persistent, mild decrease in flow, (e.g., Fig. 6).   The same dose of papaverine hydrochloride almost invariably elicited a simple, strong increase in flow lasting many minutes.

Both phases of the flow effect were beyond the bounds of statistical expectation from numerous equivalently acidic vehicle controls.   That they were not entirely secondary to the mildly altered contractility is indicated by lack of consistent quantitative relationship between the changes in contraction and flow, and by lack of the peculiar pattern of flow change in many cases of miscellaneous experimental compounds exhibiting similar effects on contraction.

A hypothetical interpretation of the negative inotropic action and the diphasic influence on coronary flow arises from the histamine-antagonizing action of many of these compounds.   Assuming that there is a release of histamine from the tissues of the heart under its unnatural circumstances, one could suppose that interference with histamine's myocardial stimulation and arteriolar constriction (rabbit heart) is followed by a masking interference with histamine's capillary dilatation.   Such an hypothesis is rendered untenable by the finding that there was no quantitative correlation between the potencies of the coronary and myocardial actions, and histamine-antagonizing actions as variously demonstrated (1, 3, 15) for many of the compounds.   Furthermore, a similar pattern of action extended into a series of several analogues of the present ethers, in which the ether function was substituted by the ester linkage, and in which histamine-antagonizing action is very poor or lacking (e.g., β-diethylaminoethyl diphenylacetate hydrochloride (18, 3, 15)).

Mild, direct negative inotropic and diphasic coronary vascular actions of BH and related compounds seem inescapable.

DISCUSSION.   The apparently linear regression of "probit" fall of blood pressure on log. of the product of dose per kgm. by its rate of injection, implies a statistical distribution of cardio-vascular elements sensitive to the drug, and accomodates cumulative statistical gradation of sensitivity both within and among units of successive, converging, physico-chemical and anatomical levels (19).   It is not surprising that both dose and its rate of injection should be factors in effective concentration of the drug for a transient effect such as the primary blood-pressure fall.   The present suitability of the logarithm of this product is in line with the well-known normalizing influence of the logarithmic transformation of concentration on statistical biological effects.

The magnitude of the vascular depression is of practical interest.   The central tendency of the present sample of data is the regression line of figure 4, computed by the method of maximum likelihood (10)[6].   The 95% confidence limits of the true central-tendency parameter for the given experimental conditions are represented by the inside hyperbolae of figure 4.   They are computed by use of the general formulation of the variance of a given probit value derived from heterog-

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[6] "k", the number of statistical units from which the individual result derives, is indeterminate in such a case.   It is set at unity.
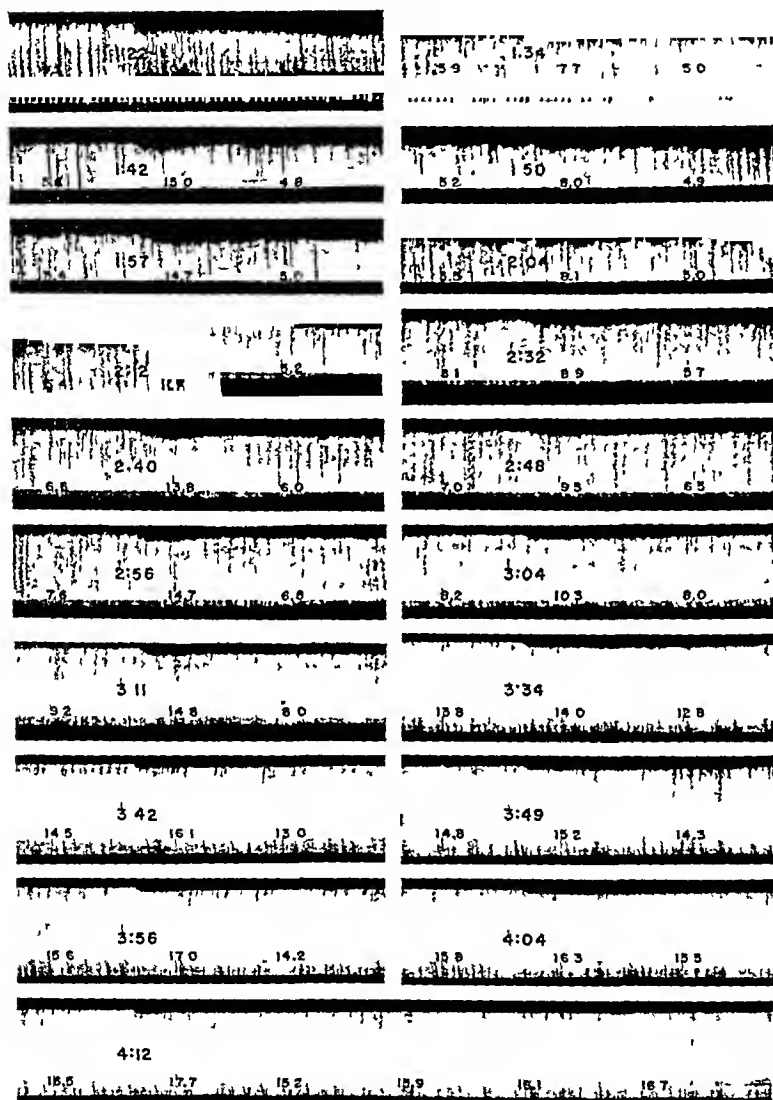
FIG 6 ISOLATED, PERFUSED RABBIT HEART, 37°C

Ventricular contractions, systole upward   Lower record (top row only), time in 5 sec
intervals, with 1 0 and 0 5 min alternately signaled   Upper figures in each section indicate
the time (P M) of injection   Lower figures indicate coronary flow in cc /min as measured
for 1 min each 1 5 min   At 1 22, 100 μgm , and at 1.42 1 57, and 2.12, 50 μgm of β piper-
idinoethyl benzhydryl ether HCl, at 1 34, 1 50, 2:04, 2 32, 2.48 3·04, 3.34, 3·49, and 4 04,
50 μgm of β-4-morpholincethyl benzhydryl ether HCl, at 2 40, 2 56, and 3.11, 50 μgm of
β-diethylaminoethyl benzhydryl ether HCl, at 3 42, 3.56, and 4:12, 50 μgm β dimethyl-
amino ethyl benzhydryl ether (Benadryl) HCl   Shortly after 2:12 it was necessary to
readjust the recording

enous sampling (10, 20)[7], on the supposition of potentially non-homogeneous sensitivity populations from animal to animal or among animal conditions.

Perhaps of more practical interest than the "average" expected effect are the 95% confidence limits within which an *individual* effect of drug injection may reasonably be expected to fall, under the experimental conditions employed. These limits are traced by the outside hyperbolae of figure 4. In their computation an estimate of the variance of the individual result must be added to that of the central tendency[8].

Set into the lower part of figure 4 are loci of products of doses by certain rates of injection. The vertical intercepts of a given dose-rate product with the confidence limits of effect, above, yield ranges of expectation for "average" or "individual" effects. For example, 1 mgm./kgm. injected at the rate 0.75 mgm./kgm./min. would be expected to yield a blood pressure fall between 0.05% and 19%, or 0.4% to 6% as the (probit) average of a large number of trials. Such a procedure (in the anesthetized dog) would thus probably be reasonably safe in the absence of critical cardio-vascular pathology. The same dose of 1 mgm./kgm. injected at the rate of 12.0 mgm./kgm./min., or 16 times as large a dose at the first rate (0.75 mgm./kgm./min.), would be expected to yield a fall between 2% and 55%, or 11% to 23% as the "average" of a large number of trials, and probably would not be regarded as conservative procedure even in the absence of cardio-vascular pathology.

Human intravenous (12, 21) to (28) dose-rate products, where calculable, and probably corresponding blood levels from ordinary oral doses, are below the lower end of the range indicated in figure 4, where effects were slight. Accordingly, there is little evidence of human "reactions" attributable to vascular depression. Cases of orthostatic hypotension (12, 21), slight lowering of systolic pressure (12, 29, 30), and faintness (21, 31, 32) may derive from an interaction of slight direct actions on the cardio-vascular system or its sympathetic control, with higher nervous actions such as sedation (cf. (30)).

The low-grade but persistent secondary rise in pressure apparently has not been described for the (unanesthetized) human subject. It was experimentally reduced to a local (non-atropinoid) and/or sympathetic vascular change. While the analogous direct secondary coronary constriction in the isolated heart suggests a *direct* local component, an *indirect* local vascular action through histamine-antagonism by BH (33, 3) under surgical experimental conditions can at least not be ruled out. The smallness of the rise, its lack of clear-cut correlation with dose, and its degradation on repetition (hence cumulation of BH injections) seem in line with such an indirect action. Variable briefness of duration, in view of prolonged maximum and median durations, cannot be interpreted as indicating

[7] $$V = \left[\frac{\Sigma Wy^2 - (\Sigma Wxy)^2/\Sigma Wx^2}{n-2}\right]\left[1/\Sigma W + x^2/\Sigma Wx^2\right],$$

where n is the number of individual results and W, the weight, is the product of a weighting factor and k. It can be shown that k factors out in the formulation, so that the present expedient of setting an indeterminate k at unity does not modify the computation.

[8] This was accomplished by adding the quantity $n/\Sigma W$ to the second factor of the formulation in the preceding footnote.

a lack of relation to the durable histamine-antagonizing action (cf. (3)). It is better viewed as a gradual compensatory or deteriorative masking, relatively selective for long-time as distinguished from abrupt vascular effects such as employed in specific demonstration of histamine antagonism.

The variable, low-grade primary cardiac acceleration occasionally observed in the presence of poor vagal tonus (phenobarbitalized dogs) is most simply regarded as manifestation of a weak atropine-like action of BH, demonstrated more specifically in antagonism of vascular depression (3) and isolated gut contraction (3, 15) elicited by acetylcholine, and suggested in certain human enteral (12), ocular (12, 31, 32, 34, 35, 36) and secretory effects (12, 21, 30–32, 35, 37–41). The threshold for this small cardiac acceleration was below that for the small opposing (direct or sympathetic-depressant) deceleration.

The very transient primary coronary dilatation in the isolated heart suggests neither liability nor asset value, and the mild secondary constriction does not resemble the immediate, intense type elicited by Pitressin[1] in similar experiments. To our knowledge no evidence of precipitation of anginal attacks by BH has appeared (cf. (42)).

The slight to moderate negative inotropic action seen in the isolated heart was repeatedly reversible.

The respiratory effects, particularly the restriction of depth, were observed mainly at supratherapeutic, intravenous, dose-rate products. It is not inconceivable, however, that occasional subjective worsening of an asthmatic condition (32, 43) might result from a tendency toward such an alteration in respiratory pattern, although no changes in human respiration were reported for non-asthmatics (12), and asthmatics are often eased.

In terms of equivalent dosage, the hydrochloride of β-4-morpholineethyl benzhydryl ether was a half or less as liable to lower blood pressure and disturb respiration as BH. It was also about a half as potent in protecting guinea pigs against histamine mist (1), less than half as potent against anaphylactic shock (2), and roughly half as toxic (5). BH, the *generally* more potent compound of the two, was selected for clinical trials. That parallelism between pharmacotherapeutic potencies and side-actions was, however, not the general rule in this series of compounds is illustrated by γ-4-morpholinepropyl benzhydryl ether. Its vascular depressant and respiratory disturbing potencies were approximately equal to those of the β-4-morpholine homologue but its protecting action against histamine mist was roughly a fourth (1).

Up to a moderately large dose-rate product (ca. 22 (mgm./kgm.)[2] /min.) BH was found to possess less depressor liability than papaverine hydrochloride; at higher products more. Relative to their blood-pressure effects, BH elicited more striking respiratory effects than papaverine.

SUMMARY

1. In the phenobarbitalized dog, intravenous Benadryl Hydrochloride (1–16 mgm./kgm., at 1–43 mgm./kgm./min.) typically elicited primary vascular depression (median duration, 2 min.) followed by rise in arterial pressure (median dura-

tion, >46 min.). Both phases were basically independent of important arterial reflex zones, vagal cardiac innervation, heart rate changes, peripheral cholinergic mechanisms, and nervous or mechanical respiratory changes. The minimal latent period was ca. 8 sec.

2. The degrees of primary fall were most satisfactorily related, as "probits" fall, with the logarithm of the product of dose per kilogram by its rate of injection. One mgm./kgm. at 0.75 mgm./kgm./min., under the conditions used, would be expected to lower blood pressure by 0.05% to 19% (95% confidence limits). The corresponding dose-rate product lies somewhat above the highest human intravenous products reviewed, and probably, in corresponding blood level, above ordinary oral doses used.

3. The secondary pressure rise was low-grade (maximum, 26 mm. Hg; median, >7 mm. Hg), decreased on repetition of injection, and was not clearly correlated with dose. With large doses it was masked by the primary depression for as long as >1.4hr.

4. Small doses were associated with a variable small cardiac acceleration (maximum, 20%) independent of blood-pressure changes, larger doses with a small primary deceleration (maximum, 22%), masking the acceleration and independent of vagus or carotid nerves. No gross disturbances in cardiac rhythm were observed in limited electro-cardiographic recordings.

5. In the isolated rabbit heart, a dose equal to one of papaverine hydrochloride which elicits slight fluctuations in ventricular contractions and strong durable increase in coronary flow, caused slight to moderate, repeatedly reversible depression of contractions, and a fleeting increase in coronary flow followed by more lasting, mild decrease in flow. The flow changes were not dependent upon mechanical changes and none of the effects was entirely dependent on anti-histamine action.

6. One mgm./kgm. frequently caused slight to moderate, transient stimulation of respiration when injected at rates of 1.2 to 43 mgm./kgm./min. One mgm./kgm. at 19 or more mgm./kgm./min. caused in addition an early restriction in depth of breathing frequently reaching temporary apnea. In such cases, and with larger doses, stimulation was expressed ultimately as acceleration or polypnea, intense and lasting well over an hour with large doses.

Vagotomy eliminated cleanly the restriction in depth and a major part of the acceleration (potentiated pulmonary proprioceptives?). Carotid denervation eliminated a weak chemo- and pressoreceptive stimulation component. In the absence of both mechanisms respiration was essentially undisturbed by large doses.

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ACTION OF DIGITALIS GLYCOSIDES ON THE CENTRAL NERVOUS SYSTEM WITH SPECIAL REFERENCE TO THE CONVULSANT ACTION OF RED-SQUILL

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It is well known that the principles of the digitalis group exert an effect on the central nervous system, and in man give rise to such manifestations as vomiting, diarrhea, headache, dizziness, drowsiness, depression, psychoses, and colored vision. It is not established whether these come about as a result of direct toxic actions on the brain cells, and there is the belief that they are produced indirectly through circulatory changes, or result from reflex stimulations (1). Hatcher and and Eggleston (2) observed hyperexcitability and other symptoms referable to the central nervous system many hours after doses of ouabain and digitoxin in the rat. Weiss (1) stated that external stimuli occasionally produce strychnine-like convulsions in cats after digitoxin and other digitalis preparations. Straub (3) observed convulsions after the aglycone, digitoxigenin, in cats and frogs. Chen et al. (4) described convulsions followed by prostration after digitoxigenin in the cat and frog, and after digoxigenin in a cat, and since corresponding doses of the glycosides failed to produce these effects, they concluded that the sugar in the molecule diminishes the action on the central nervous system. Cattell and Gold (5) also observed camphor-like convulsions after intravenous doses of digitoxigenin in unanesthetized cats, which were unrelated to the action on the heart and were prevented by anesthesia; intravenous digoxigenin in cats caused ventricular fibrillation without convulsions except for the terminal asphyxial fit after the heart stops, which is known to occur in the case of all digitalis glycosides.

A central sedative action has been ascribed to adonis glycosides (6) and experiments showing protection against convulsions in rabbits by cocaine, camphor, and picrotoxin have been reported (7). This hypnotic and anticonvulsant action has been ascribed to a special glycosidal fraction of adonis (8).

Of the two varieties of squill, the red and the white, the white squill is the one most commonly employed for its digitalis action in therapeutics. The red squill is better known for a convulsant action in rats and has long been extensively employed as a rodenticide (9). From the difference in the behavior of white and red squill by oral administration in rats, Winton (10) concluded that the cardiac glycosides and rat-poisoning principles in red squill are not the same. Several years later, Wokes and Willimott (11) stated that "it is now well known that the toxic effect of red squill when given orally is not due to the specific cardiac glycosides."

There is a fairly extensive literature dealing with the practical aspects of the problem of red squill as a rat poison, but there is a dearth of critical pharmacologic investigation. Most of the comparisons of red with white squill in the rat are based on oral administrations, and the view that the convulsant action in rats is due to some factor other than the cardiac glycoside seems to prevail.

The present study was undertaken to explore the pharmacology of the convulsant principle in red squill, and to compare it with known glycosides of the digitalis series. The results (preliminary report, 12) indicate that the convulsant action of red squill is not due to a special factor but is produced by the cardiac principle, that, in rats, an action on the central nervous system giving rise to a mixed picture of convulsions and paralyses is a property common to the glycosides of the digitalis group, and that this central action is more highly developed in some of the glycosides than in others.

EXPERIMENTAL. Three materials from squill were utilized in this study. They were all very potent but impure. One was "amorphous scilliroside," a fraction[1] obtained from a red squill powder by a procedure described by Stoll and Renz (13). Another extract from red squill was obtained from the Lederle Laboratories, under the name of urginin[2] (The Grissard Laboratories). The former powder is light yellow, and the latter somewhat more brownish. A specimen of white squill glycoside used for comparison was also obtained from the Lederle Laboratories under the name of scillicin (Italian).

All intravenous injections were made in the tail vein. With few exceptions, the Wistar strain female rats were used. It is stated that the toxic dose for the female is one-half that for the male (14). Since most of the active materials required alcohol for solution, the effect of intravenous alcohol was tested in each of 3 rats in doses of 0.25, 0.4, and 1.0 cc. of 95 per cent alcohol per kg. The alcohol was injected in concentrations of 5 to 25 per cent. Following the largest dose the animal showed immediate depression and rolled over directly after the injection. The effects wore off within 2 minutes. The smaller doses produced no effects. The amount of alcohol given with the doses of the drug usually represented less than 0.1 cc. of 95 per cent alcohol per kg. In the case of the less soluble materials requiring very large doses of the glycoside, there were a few instances in which the dose of alcohol was as high as 1 cc. per kg. The description of the effects of the drugs is based on those cases in which the dose of alcohol was far below that which causes symptoms.

AMORPHOUS SCILLIROSIDE IN RATS. *General effects.* The course of poisoning was first studied after intravenous injections. In its most typical form seen after the intravenous LD50 (1.25 mg. per kg) (table 1), the effects are produced in three stages; namely, a brief initial "shock" effect, followed by a long asymptomatic interval, followed in turn by a prolonged convulsant phase.

The initial effect occurs immediately after the injection. There is marked weakness and ataxia. It lasts about 10 minutes or less. With larger doses, there is immediate collapse with general depression, depressed respiration, the animal lying sprawled out or on its side and unable to move. The heart is rapid and regular during this period. Sometimes there was doubt as to whether the animal would survive because of the severity of the prostration. However, recovery invariably begins almost at once, within 2 or 3 minutes the animal is again running about; and within 10 minutes or less, the appearance and behavior are quite normal. This initial "shock" effect of the intravenous injection may be due to the high concentration of the drug in the blood stream, suggested by the fact that it is so

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[1] It was supplied by C. R. Noller of Stanford University, Sample Nos. Stan. U.-278 and 285. In a personal communication, Dr. Noller estimated the material to be about 60 per cent pure.

[2] The "urginin" of commerce is prepared from white squill.

fleeting. Whether it is the result of an action on the central nervous system, the vasomotor center, the blood vessels directly, or the heart, or some other site is not known.

After an interval of 2 to 3 hours or longer during which the animal seems normal, the second phase of the poisoning begins and progresses at varying rates depending on the size of the dose. These symptoms have the appearance of curarization. There is muscular weakness, more marked in the hind limbs. The limbs tend to sprawl out. There is ataxia. Agitation appears, the animal thrashing and whirling about on the long axis using the hind limbs as a pivot. There is nodding of the head and tremulous movements of the limbs and head. Reflex hyperexcitability develops and within about 2 hours, convulsions. Sometimes the convulsion is brought on by stirring the animal; at other times, it occurs spontaneously. The convulsions sometimes resemble the tetanus of strychnine poisoning and at other times the myoclonic fit of camphor. There is labored and irregular respiration, urination, and salivation. A long series of violent convulsions sometimes develop with the animal rolling over and over many times. The heart beat is strong and usually very

TABLE 1

Toxicity of amorphous scilliroside by intravenous injection in rats

DOSE	NO. OF RATS	NO. WITH CONVULSIONS	NO. DIED WITH CONVULSIONS	NO. DIED WITHOUT CONVULSIONS
mg. per kg				
0.25	2	0	0	0
0.4	6	0	0	0
0.5	6	3	1	0
0.75	8	4	0	0
1.0	11	5	4	0
1.1	10	4	4	0
1.25	10	6	5	0
1.5	2	2	2	0
2.0	1	1	1	0
3.0	3	0	0	3
5.0	4	0	0	4
10.0	1	0	0	1

rapid. The convulsive symptoms are associated with muscular weakness, and periods of convulsions alternate with marked generalized muscular depression. After 2 or 3 hours in this state, a sudden convulsion is followed by paralysis of respiration. At postmortem, the heart is found beating at slow rates of about 60 a minute; there are no gross pathological changes.

After smaller convulsant doses, the initial "shock" effect is milder, the latent period to the appearance of convulsions is longer (about 10 hours or more), and after a period of convulsions of varying duration, sometimes days, the animal dies or recovers. Survivors continue to show ataxia and weakness for as long as a week or two.

Still smaller doses produce muscular weakness, hyperexcitability, agitation, and tremulous movements but no convulsions.

Minimal effects result from an intravenous dose of 0.25 mg. per kg. In such cases, there is a slight initial depression which wears off within about 1 or 2 minutes. The animal then appears normal for about 4 hours, when slight weakness and ataxia appear. These usually persist for many hours, faint weakness being present in some cases for more than 24 hours.

When the intravenous dose of scilliroside is increased to the range of about twice the convulsant LD50 or larger, a different type of poisoning appears (table 1). The injection is followed almost immediately by symptoms of extreme prostration with labored respiration,

feeble and irregular heart, and death with cessation of heart beat without convulsions within a period of a few minutes. When the chest is opened immediately, the heart is found in stand-still and dilated, or in ventricular fibrillation (fig. 1). This is indistinguishable from the cardiac death produced by the typical digitalis glycosides by intravenous injection in rats.

It is clear that scilliroside may cause death by either of two mechanisms in the rat, one, an action on the central nervous system, and the other, an action on the heart. There remains the possibility that either or both may be complicated by direct vasomotor actions.
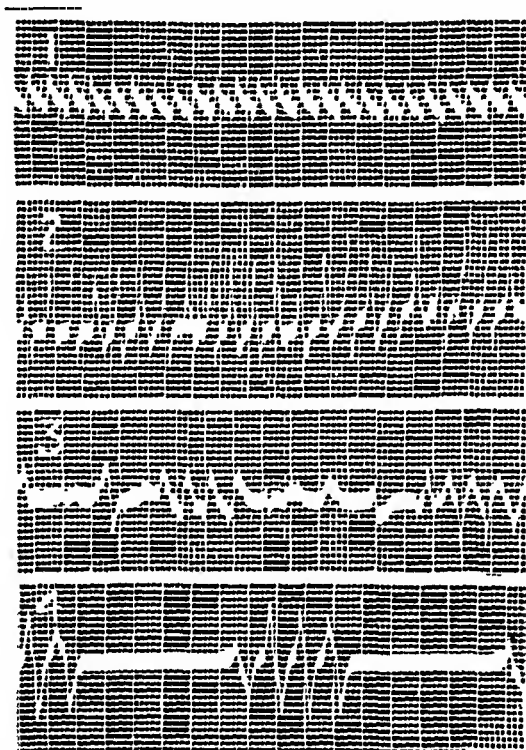
FIG 1. Electrocardiograms (Lead 2), rat no 186, female   (1) control, (2) 10 sec after 5 mg /kg. amorphous scilliroside intravenously; (3) 45 sec after dose, (4) 1 min after dose Cardiac standstill immediately after last electrocardiogram

The two mechanisms are distinguishable by their symptoms and course; the convulsant action requires hours to develop and the course is fairly long, lasting many hours to many days; the cardiac action appears promptly and the course is quite brief, resulting in death or recovery in periods of a few minutes to a few hours   The tolerance of the two structures show wide differences, and in view of the fact that the more sensitive mechanism takes hours to develop its action (convulsant), and the less sensitive takes only minutes (cardiac), it is possible to establish the LD50 for each. As seen in table 1, the intravenous LD50 for scilliroside in the rat is 1 25 mg by the convulsant action, and about 2 5 mg. per kg. by the cardiac action.

The course of poisoning with scilliroside by intramuscular injection and oral administration is essentially similar to that by intravenous injection. The initial "shock" effect of the intravenous injection is usually absent although frequently a transient depression appears within 5 to 10 minutes. The convulsant action runs a course of hours and the cardiac action, of a few minutes. The effects of fatal oral doses of red squill have been described by others (15, 16). The delay of several hours in the development of the convulsant action even after intravenous injection, interferes with the use of the onset of effects as a means of determining the speed of absorption of scilliroside; after intramuscular or oral

TABLE 2

Toxicity of amorphous scilliroside by intramuscular injection in rats

DOSE	NO OF RATS	NO WITH CONVULSIONS	NO DIED WITH CONVULSIONS*	NO DIED WITHOUT CONVULSIONS
mg. per kg				
0 3	2	2	1	0
0.4	2	2	1	0
0 5	4	3	2	0
0 6	1	1	1	0
0 7	1	0	0	0
0 8	1	1	1	0
0 9	1	1	0	0
1 0	2	2	2	0
2 0	1	1	1	0
3 0	2	2	1	0
4 0	2	2	2	0
5 0	4	3	3	1
6 0	3	3	3	0
7 0	2	2	2	0
8 0	2	2	2	0
10 0	2	2	2	0
15 0	2	2	2	0
20 0	2	2	2	0
25 0	2	2	2	0
30 0	3	2	2	1
40 0	4	3	3	1
44 0	1	1	1	0
50 0	2	0	0	2
100 0	2	0	0	2

* In the fatalities after the small doses of 0 3 to 0 8 mg , the rats had prolonged convulsions and were found dead in the morning of the fourth to sixth day They took no food or water during this period, hence, the cause of death may not have been the direct action of the drug

doses which cause death by the convulsant action, the rat may appear normal for several hours The larger doses which cause death by the cardiac action, however, indicate that scilliroside is quite promptly absorbed from the muscle, effects may appear within a matter of 5 minutes after an intramuscular dose of 5 mg per kg An oral dose of 10 mg. per kg. produced effects in an hour

The LD50 by the 3 methods of administration has not been determined with precision. The indications are that the convulsant LD50 by intramuscular injection (table 2) is of the same order as that by intravenous injection. There is indication that the oral dose is about twice that of the intravenous The cardiac LD50 by intramuscular injection, however, is

about 10 times the intravenous dose. This suggests rapid elimination and poor fixation of the drug by the heart muscle.

It is noteworthy that, by the convulsant action on the central nervous system, it usually takes from 4 to 6 hours to kill the rat with scilliroside, irrespective of the size of the dose or the route of administration, although in a few instances intramuscular massive doses, 25 mg. per kg. or larger (about 20 times the LD50), proved fatal by the convulsant action after as short a period as 1 hour.

AMORPHOUS SCILLIROSIDE IN FROGS. Scilliroside was injected into the ventral lymph sac in concentrations of 1:2000 to 1:4000 in each of 7 frogs in doses of from 0.1 to 1.0 mg. per kg. There were no signs of stimulation or convulsions. The frogs survived doses below 1 mg. during a 3-day period of observation. The 1-mg. dose produced depression and death with the heart in systolic standstill typical of the digitalis glycosides.

AMORPHOUS SCILLIROSIDE IN CATS. *General effects*. The effect of scilliroside by intravenous injection in unanesthetized cats was observed in each of 4 animals which received the following doses: 0.1, 0.15, 0.2, and 1.0 mg. per kg. The smallest dose caused almost immediate panting with recovery. The remaining doses caused panting, signs of nausea, vomiting, diarrhea, prostration, tachycardia, sometimes preceded by marked cardiac slowing, terminal asphyxial convulsions and death within periods of from 5 to 7 minutes.

TABLE 3
Comparison of amorphous scilliroside and scillicin by oral administration in cats

CAT NO.	DRUG	DOSE		TIME TO ONSET OF		
		Cat units	Total	Nausea	Vomiting	Diarrhea
			mg			
8	Scilliroside	5	1.9	145 min.	145 min.	
9	Scilliroside	10	4.9	26 min.	26 min.	122 min.
10	Scilliroside	15	5.7	130 min.	130 min.	135 min.
11	Scillicin	5	3.2	27.5 hrs.	27.5 hrs.	
12	Scillicin	10	7.5	84 min.	0	
13	Scillicin	15	10.6	40 min.	40	133 min.

There were no heart sounds at the time of the convulsion. These effects are characteristic of the digitalis glycosides.

The effects of scilliroside by oral administration in cats were observed in each of 3 animals which received 5, 10, and 15 cat units per kg. respectively. The drug was administered on an empty stomach by means of the stomach tube and washed down with 20 cc. of water. An electrocardiogram was taken before the drug and again 24 and 48 hours later. There were no changes in the electrocardiogram indicative of action on the heart. The results are summarized in table 3. The drug caused fairly prompt signs of nausea, vomiting, and diarrhea, probably due to a local action. One cat showed slight ataxia which began in about 5 hours and lasted about 18 hours. There was some weakness in association with the violent retching, vomiting, and diarrhea which lasted several hours. All animals seemed normal within 24 to 48 hours. Gastrointestinal absorption of scilliroside is poor in the cat, although, as already indicated, absorption is rapid in the rat.

Papillary muscle. Scilliroside was tested on the systolic force of the papillary muscle of the cat's right ventricle by the method of Cattell and Gold (17). The compound was added to the fluid bathing the muscle in a concentration of 1:5,000,000. Within a few minutes, the systolic force began to rise and reached a peak of nearly 5 times that of the control (1.5 cm. to 7 cm.). An increase in the concentration to 1:1,000,000 resulted in poisoning with loss of excitability within about 15 minutes. This effect on the papillary muscle is characteristic of all the digitalis glycosides.

Cat unit potency. The potency of scilliroside by intravenous injection in cats was tested by the technic used in the USP XII method of bioassay of digitalis. The results in 6 cats showed an average cat unit potency (LD50)[3] of 0.133 ± 8.1% mg. per kg. for sample No. 278 and 0.162 ± 5.4% for No. 285.

Effect on the blood pressure. The effect of scilliroside on the blood pressure was tested in 1 cat during local procaine anesthesia. The material was injected intravenously in a concentration of 1:125,000 in small doses at intervals of 5 minutes until death which occurred with terminal convulsions within a period of 95 minutes. Blood pressure records were obtained from the carotid artery at frequent intervals. Figure 2 shows the effects on the blood pressure. This is fairly typical of the effect of digitalis glycosides on the blood pressure of the cat, namely, progressive fall with sudden collapse at the time of the ventricular fibrillation.

Effect on the electrocardiogram. The effect of scilliroside on the electrocardiogram was examined in 1 cat which received a total of 0.15 mg. per kg. in 2 doses by intravenous injection. This caused death with terminal convulsions in a period of 55 minutes. Selected sections of the tracings (Lead 2) taken at intervals throughout the course of the poisoning
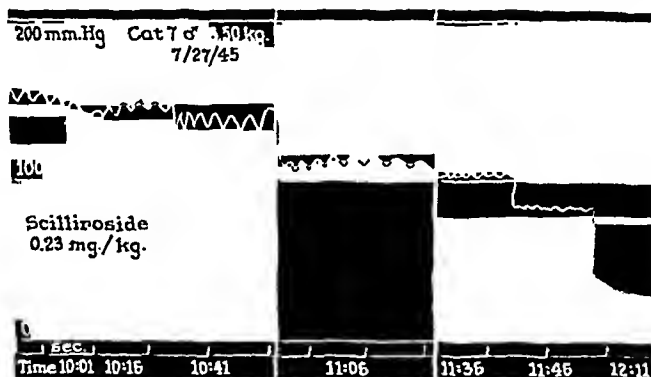
FIG. 2. EFFECT OF AMORPHOUS SCILLIROSIDE ON BLOOD PRESSURE OF CAT

are reproduced in figure 3. They show the changes in T-wave, ventricular tachycardia, and terminal ventricular fibrillation, characteristic of the digitalis glycosides.

AMORPHOUS SCILLIROSIDE IN MAN. Five patients with auricular fibrillation and a rapid ventricular rate were put to bed. They had been without digitalis for 3 weeks. The apex rate was counted several times daily during a control period of several days until the rate was stabilized. Each received a single oral dose on an empty stomach. The rate was counted at frequent intervals during the day and 3 times daily during the subsequent days in order to ascertain the curve of development of effects and their disappearance. Each of the 5 received a single oral dose of 0.4 mg. (3 cat units), a week later 4 times the dose (1.6 mg.), and a week thereafter, 4 of them received 8 times the dose (3.2 mg.). When all effects disappeared, each of the 5 patients received a single intravenous dose of 0.4 mg. The average effects on the ventricular rate are charted in figure 4. The results show that amorphous scilliroside by intravenous injection produces a slowing of the ventricular rate in patients with auricular fibrillation, characteristic of the digitalis glycosides. The full action develops promptly in a period of about 1 hour. The elimination is rapid, the effects disappearing within about 4 to 5 days. In these respects, scilliroside resembles the action of ouabain. Like other glycosides of squill, it is poorly absorbed after oral administration, a dose 8 times

[3] By the method of injection used in the U.S.P. XII assay of digitalis.

the full digitalizing intravenous dose producing only a very slight and transient slowing of the heart rate.
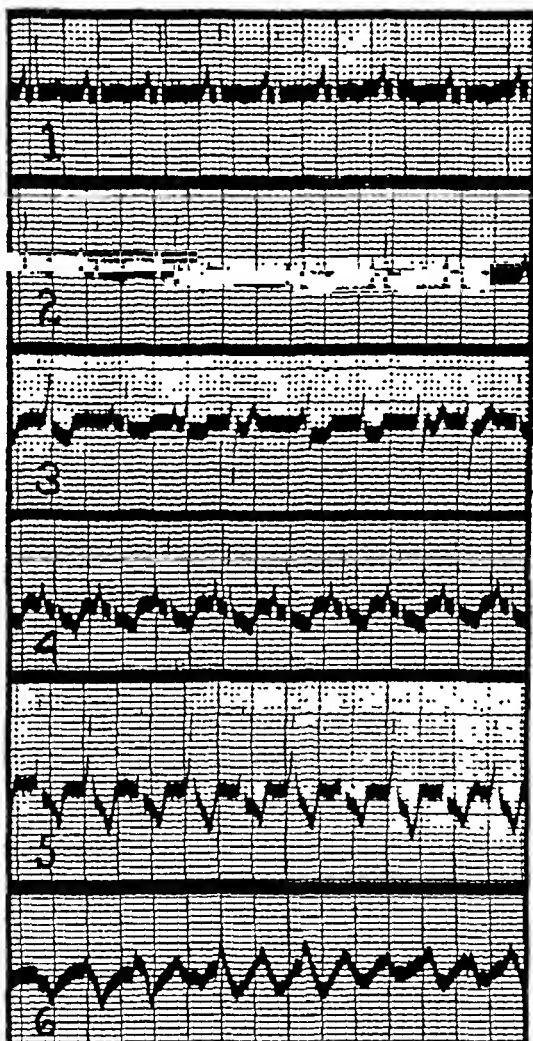
FIG. 3. Electrocardiograms (Lead 2) of cat no 5   (1) control, (2) 5 min after 0 075 mg./kg. amorphous scilliroside intravenously, (3) 24 min after dose, (4) 35 min. after dose; (5) 1 min. after second dose of 0 075 mg and 1 hr. after first dose; (6) 5 min. after second dose; convulsion

Each of 3 patients with normal sinus rhythm received intravenous doses of amorphous scilliroside, 3, 3, and 4 cat units respectively, for the effects on the electrocardiogram.

In the one with the largest dose, there occurred, within 1 hour after the injection, depression of the T-wave characteristic of digitalis action.

SCILLICIN (WHITE SQUILL) IN RATS. Claremont (9) stated that the red and white squill are botanically identical and difficult to distinguish. Their oral toxicity in rats has been suggested as the only reliable means of distinguishing them (9, 11). Munch et al. (18) stated that the white squill "does not kill rats." Winton (10) found that the two types of squill are about equally potent by injection in frogs, but by oral administration in rats there is a very marked difference, the red squill showing an LD50 of about 620 mg. per kg., and the white squill producing no effect in a dose about 7 times as great. LeBlanc and Lee (19) observed no effect in rats fed white squill in doses 16 times the fatal dose of red squill. From a few experiments with an extract of the two squills by subcutaneous injection in rats, Winton (10) suggested that the red squill is about 6 times as potent as white by injection. Wokes and Willimott (11) confirmed the observation that the two types of squill have approximately equal potencies in terms of their cardiac action by lymph sac injection in frogs,
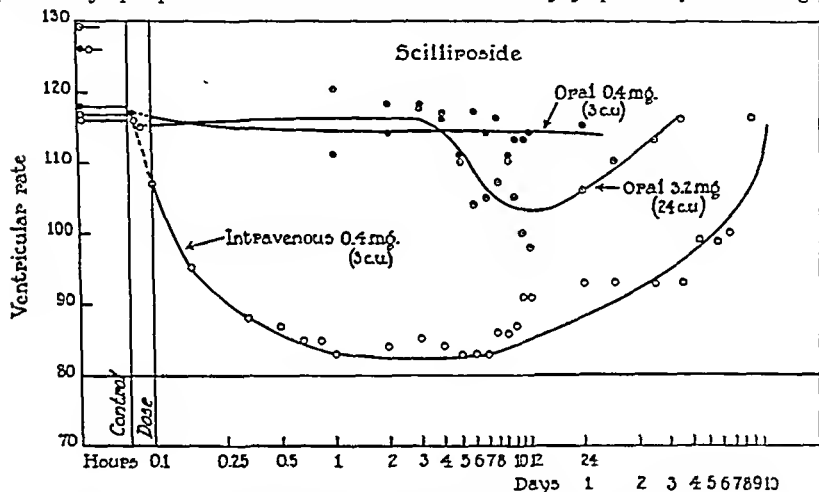
FIG. 4. EFFECT OF AMORPHOUS SCILLIROSIDE ON THE VENTRICULAR RATE IN PATIENTS WITH AURICULAR FIBRILLATION
The curve for the 0.4-mg. oral dose and the intravenous dose represents the average response in 5 patients; the 3.2-mg. oral dose, in 4 patients.

and also observed that, while the white squill powder is toxic to rats, it requires from 10 to 15 times as much by oral administration. There is no clear statement regarding a convulsant action of white squill in rats, although the suggestion has been made that a small amount of the convulsant factor may be present in white squill.

Experiments were carried out in the rat with scillicin, a water-soluble amorphous glycosidal material obtained from Italian white squill, which had been investigated in the cat and dog by Wallace and Van Dyke (20).

Tables 4 and 5 show the effects of scillicin by intramuscular and intravenous injection. The white squill preparation was less potent than scilliroside by both routes, the smallest lethal dose being of the order of 10 mg. per kg. by the intravenous route and 25 mg. by the intramuscular route. By intramuscular injection, scillicin produced effects essentially similar to those of scilliroside. The smaller doses cause death by the convulsant action, and the larger doses by the cardiac action (table 4). However, by intravenous injection, it was not found possible to elicit the convulsant action (table 5); the animal either died within a few minutes from the cardiac action, or recovered from the muscular weakness

within a few hours The white squill preparation, therefore, possesses a convulsant action, but the ratio of convulsant to cardiac actions is different from that of the scilliroside In the red squill by intramuscular injection, the ratio of the convulsant to the cardiac potency is about 30:1 while in the white squill, it is about 5:1. It cannot be decided from these results whether the difference in the ratios is due to a difference in molecular structure of the two materials, or to the admixture of a small amount of red squill glycoside in the preparation of the white squill, a possibility already suggested by others. If the white squill material contained as little as 0.5 to 1.0 per cent of scilliroside, it could account for

TABLE 4

Toxicity of scillicin by intramuscular injection in rats

DOSE	NO OF RATS	NO WITH CONVUL-SIONS	NO DIED WITH CON-VULSIONS	NO DIED WITHOUT CONVULSIONS
mg. per kg.				
10	2	0	0	0
20	2	0	0	0
30	1	1	1	0
40	1	1	1	0
50	1	1	1	0
100	2	2	2	0
150	1	0	0	1
200	1	0	0	1

TABLE 5

Toxicity of scillicin by intravenous injection in rats

DOSE	NO OF RATS	NO. WITH CONVUL-SIONS	NO DIED WITH CON-VULSIONS	NO DIED WITHOUT CONVULSIONS
mg. per kg.				
1.0	1	0	0	0
1.5	1	0	0	0
5 0	1	0	0	0
10.0	4	0	0	1
12.0	1	0	0	1
15 0	1	0	0	1
18 0	1	0	0	1
25 0	1	0	0	1
30.0	1	0	0	1
50.0	1	0	0	1
100 0	1	0	0	1

the convulsant action in the intramuscular doses and the absence of a convulsant action in the intravenous doses of the scillicin.

Urginin (Red Squill). This is a purified alcohol-soluble glycosidal material obtained from red squill. Experiments were performed in 19 rats by intravenous and oral administration. It produced the convulsant action regularly and the course of poisoning was similar to that of scilliroside. Oral doses of 20 mg. per kg. or more proved fatal by the convulsant action. An intravenous dose of 2 2 mg per kg. proved to be an LD30, showing, therefore, a toxicity of approximately one-half that of scilliroside by the convulsant action in rats. The cat unit potency of this specimen of urginin was 0 252 mg per kg , or approximately one-half the potency of scilliroside in cats. The fact that one of these two materials

from red squill which was twice as potent as the other by the cardiac action in cats, was also twice as potent by the convulsant action in rats, suggests that the cardiac and convulsant actions run parallel in the process of purification.

Other Digitalis Glycosides in Rats. In view of the foregoing observations pointing to the probability that the action on the central nervous system of rats by red squill is exerted by its cardiac principle, experiments were performed in the endeavor to determine to what extent this action in rats is produced by other members of the digitalis group.

Digitoxin. A specimen of digitaline Nativelle was employed. This was administered to each of 8 rats. Convulsions typical of red squill occurred in 4 (3 by intravenous injection and 1 by oral administration). The convulsant doses used (vein) were 3, 3, and 5 mg. (7.5 to 12.5 cat units) per kg. and the oral dose was 50 mg. (125 cat units) per kg.

An intramuscular dose of 5 mg. caused weakness and hyperexcitability in 1 rat with recovery, and death without convulsions in another. After the intravenous doses the animals developed the initial "shock" effect lasting a few minutes with recovery, and then convulsions in from 1 to 3.5 hours. The convulsant periods lasted 0.5 hour, 3.5 hours, and 27 hours, and death occurred in from 4 hours to 3 days after the injection. Of the 3 rats with oral doses of 50 mg., 2 showed little absorption with slight weakness and recovery during a period of 2 days; the third developed weakness in about 3 hours, violent convulsions in 27 hours and died 30 hours after the dose.

Ouabain. A specimen obtained from Merck and Co. was employed. This glycoside given by a single intravenous injection to each of 7 rats in doses of from 1 to 25 mg. (10–250 cat units), average 11 mg. (110 cat units) per kg. failed to cause convulsions. Doses of 2 mg. or larger produced the initial "shock" effect (falls over, weak, staggers) lasting a few minutes. After the larger doses, the brief recovery was followed by a secondary period of muscular weakness, the animal being unable to stand, legs sprawled, snakelike movements of the body in efforts to walk, resembling curarization, and respiratory difficulty. There were no signs of narcotization, the animal appearing alert. After doses up to 16 mg., recovery progressed fairly rapidly and the animals appeared normal within about 2 hours or less. One rat, after 16 mg., showed marked unrest with reflex hyperexcitability. A dose of 20 and 25 mg. proved fatal in 2 to 2.5 minutes. In these also the initial "shock" effect was followed by fleeting recovery, and then within a minute sudden cardiac arrest which proved fatal.

These experiments suggested that the rapid elimination might account for the absence of effects on the central nervous system. Accordingly, single doses of from 40 to 50 mg. per kg. were given intramuscularly to each of 5 rats. The course of poisoning was longer; one died with marked depression within 1.25 hours; the remaining 4 survived periods of poisoning of from 8 to more than 24 hours. Two of these developed marked hyperexcitability in from 2.5 to 7 hours.

Time seemed to be a factor in eliciting the action of ouabain on the central nervous system. One rat received doses of 10 and 20 mg. per kg. intramuscularly at 30 minute intervals for 14 doses, total 180 mg. per kg. in 6.5 hours; this caused cardiac death with ventricular fibrillation, but during the course of the poisoning marked action on the central nervous sytem developed with spacticity, paralysis, and convulsive strychnine-like responses. In each of 2 rats which received an intramuscular injection of 40 mg. per kg., a second injection of 5 mg. per kg. intravenously 5 hours later produced fairly typical convulsions in about 30 minutes; both survived.

Each of 2 rats which received 200 mg. per kg. orally, showed no effects.

Gitalin. A specimen obtained from Rare Chemicals, Inc. was employed. This was administered intravenously to each of 3 rats in single doses of 10, 25, and 50 mg. (17, 42, and 85 cat units) per kg. The course of poisoning was similar to that of the red squill principle. The one with the smallest dose showed no convulsions and survived. After the 25 mg. dose, violent convulsions developed in 7 hours and lasted about 20 hours. This was followed by profound depression and paralysis with death in 3 days after the injection. The 50 mg. dose caused death in 1.25 hours; there were brief convulsive seizures associated, however, with marked cardiac irregularities.

Folinerin. This glycoside obtained from oleander (Schering Corporation) produced no convulsions after single intravenous doses of from 2 to 20 mg. (9.5 to 95 cat units), average 7.6 mg. (36 cat units) per kg. in each of 12 rats. Five of these died of cardiac action within from 1 to 3 minutes and after doses of 5 to 20 mg. The survivors (doses 2 to 10 mg.) showed the immediate "shock" effect and the secondary stage of muscular weakness with apparent recovery in periods of from 5 to 24 hours.

A variety of other doses and routes of administration were tested in 10 additional rats, arranged to produce more prolonged periods of poisoning, such as daily intravenous doses for from 7 to 9 days (total 34 to 49 mg. per kg.), daily intramuscular doses for from 6 to 12 doses (total 600 mg. per kg.), daily oral doses for 5 days (total 1000 mg. per kg.), intramuscular dose of 50 mg. per kg. followed on the next day by 5 mg. per kg. intravenously. Three of these produced convulsant poisonings, typical of red squill; in one, after an intravenous dose of 5 mg. per kg. preceded by 6 daily intramuscular doses of 100 mg. per kg. each; in another, after an intravenous dose of 5 mg. per kg. following 50 mg. per kg. intramuscularly

TABLE 6

Convulsant activity of several members of the digitalis group by intravenous injection

DRUG	CONVULSANT ACTIVITY IN RATS	LD50 IN CATS	LD50 IN RATS	MODE OF DEATH BY M.L.D. IN RATS
		mg. per kg.	*cat units per kg.*	
Scilliroside (red squill) .	++++	0.133	9.4	convulsant
Urginin (red squill)	++++	0.252	±10	convulsant
Scillicin (white squill)	+++?	0.215	±56	cardiac
Digitoxin (Dig. purp)	++++	0.42	7.5*	convulsant
Gitalin (Dig. purp.)	++++	0.59	±30*	convulsant
Ouabain	+	0.100	±200*	cardiac
Folinerin (oleander) .	++	0.214	±50*	cardiac
Digoxin (Dig. lan.) .	?	0.28	36*	(no deaths)
Lanatoside C (Dig. lan.) .	?	0.25	20*	(no deaths)
Digilata (Dig. lan.)	?	1.10	45*	(no deaths)
Convara (convallaria)	?	—	±50*	cardiac

* Rough approximations.

24 hours previously; in the third, after an intravenous dose of 2 mg. per kg. preceded by 5 daily oral doses of 200 mg. per kg.

Digoxin. The purified glycoside from digitalis lanata, obtained from Burroughs Wellcome and Co., was employed. This failed to cause convulsions in each of 5 rats which received from 1 to 10 mg. (3.6 to 36 cat units) per kg., average 4.6 mg. (16.4 cat units) per kg. by intravenous injection. They all survived and showed apparent complete recovery in periods up to approximately 4 hours.

Lanatoside C. Cedilanid (Sandoz Chemical Works) obtained from digitalis lanata was employed. Each of 2 rats received 2.5 and 5 mg. (10 and 20 cat units) per kg. intravenously. Only the larger dose caused weakness and ataxia with recovery in 45 minutes. There were no convulsions.

Digilata. This purified extract of digitalis lanata, obtained from the Harrower Laboratory Inc., was injected intravenously in each of 4 rats in doses of from 10 to 50 mg. (9 to 45 cat units) per kg., average 30 mg. (27 cat units) per kg. There were no convulsions and signs of poisoning disappeared in an average period of about 8 hours.

Convara. The material, an impure extract of convallaria, obtained from George A. Breon and Co., was injected intravenously in each of 6 rats in doses of from 25 to 100 cat units, average 50 cat units per kg. Doses of 50 cat units or more proved fatal in periods

up to 2 minutes by cardiac action. The 3 survivors developed weakness and ataxia, and showed apparently complete recovery in an average of about 15 hours. There were no convulsions.

Table 6 summarizes the data on the various materials used in the study showing their relative convulsant activity. The relative potencies of various digitalis glycosides differ in the cat from those in the rat. This is due in part to differences in the sensitivity of the heart in the two species to different compounds, and, in part, to the fact that several of them cause death in the rat by an action on the central nervous system before enough of the drug is present to poison the heart.

DISCUSSION. It is well-known that red squill contains cardiac glycosides of the digitalis series, and that it also exerts a conspicuous convulsant action in rats. The convulsant action by oral administration in rats has led to its extensive use as a rodenticide. The view prevails that the convulsant action in rats is due to a specific principle distinct from the cardiac glycosides. Several points favored this view, namely, the fact that minute doses cause the convulsant action (as small as 0.3 mg. per kg. of the purified material used in this study) while the rat is known to be very tolerant to the cardiac action of the digitalis glycosides, the fact that the convulsant action lasts several days while the rat is known to recover rapidly from the cardiac action of the digitalis glycosides, and the fact that the convulsant action appears after very small oral doses while the rat is known to absorb digitalis glycosides so poorly that it is difficult to kill the rat by the oral administration of these materials. The evidence for a distinct convulsant principle in red squill, however, was not sufficiently convincing. Accordingly, the matter was re-examined in the present investigation.

For this purpose a highly purified material of red squill, amorphous scilliroside, was studied. It was one of the most potent preparations of red squill from the standpoint of a convulsant action in rats. This was found to possess also the typical actions of a cardiac glycoside in the rat, frog, cat, and man. The convulsant action occurred only in the rat. The fact that the material is not a single and pure principle left open the possibility of the mixture of a cardiac and a convulsant principle, but against it is the high cardiac potency of the material in the cat and man; in them it is the most potent of the cardiac glycosides of squill possessing an activity of the order of ouabain. Another material obtained from red squill, urginin, which was found to be one-half as potent as amorphous scilliroside by the convulsant action in rats, also proved to be one-half as potent by the cardiac action in cats. The convulsant and cardiac activities of red squill, therefore, seem to run parallel in the process of extraction indicating that the two actions belong to one principle. The most decisive proof awaits the repetition of these experiments with a pure principle of red squill.

The inference that the convulsant and cardiac principles are identical, received further support from the observation that several other glycosides of the digitalis group cause convulsions in the rat, digitoxin, gitalin, folinerin, and ouabain. While amorphous scilliroside in suitable doses, uniformly caused convulsions, the convulsant action was elicited with varying degrees of regularity in the case of the other members of the digitalis group. A limited number of experiments with digitoxin indicates that it causes convulsions in the rat with substantially the

same regularity as in the case of scilliroside. Scillicin, a water-soluble glycosidal material from white squill regularly caused convulsions by intramuscular injection in the rat, and death by the cardiac action after a dose about 5 times as large. However, the white squill material failed to cause convulsions after intravenous doses; in these cases the rat either recovered or died of the cardiac action within a few minutes. The evidence leaves open the possibility that the convulsant actions of scillicin may be due to admixture of small amounts of red squill. In the case of ouabain and folinerin, it was found that while single doses failed to cause convulsions, repeated doses often brought on a convulsant state. One of the effects of repeated doses was to produce a sustained period of poisoning allowing for the development of the convulsant action which requires several hours.

It is clear that to produce convulsions in the rat is a property common to the digitalis glycosides. Whether some of the digitalis glycosides are devoid of this action cannot be stated, since those which failed to produce convulsions were not studied in sufficient detail. It is more difficult to elicit this action in the case of glycosides which the rat eliminates very rapidly (ouabain). It also appears that the convulsant factor is not equally developed in the molecular structure of the different glycosides. This is especially manifest in a comparison of ouabain with scilliroside; even severe poisoning long maintained by repeated doses of ouabain often failed to cause the typical convulsions regularly produced by scilliroside, and the amount of ouabain required to kill by the cardiac action was smaller than that by the convulsant action, whereas the reverse was the case with amorphous scilliroside.

How the cardiac glycosides bring about the camphor-like or strychnine-like convulsions in rats is not known. The convulsions are mixed with profound muscular depression and prostration resembling curarization. They are clearly not the result of an action on the heart because during the period of hours or days in which they occur, the heart beat is strong and rapid, and the electrocardiogram shows no significant changes. They are not to be confused with the terminal asphyxial fit produced by digitalis as the result of ventricular fibrillation commonly seen in the cat and dog. The long latent period even after massive intravenous doses suggests that it may not be a direct action of the glycoside but of a decomposition product, or that the effect is due to a secondary metabolic or structural change in the central nervous system requiring hours to bring it about, irrespective of the dose.

It has already been pointed out that the convulsant action of digitalis glycosides is not confined to the rat, that some action on the central nervous system is obtained in other species, and that such convulsions occasionally occur in cats. Several factors may account for the regularity with which convulsions are produced in the rat as compared with other species, such as, differences in fixation or rate of elimination, differences in relative susceptibility of the heart and central nervous system in different species, and the high tolerance of the rat's heart to the digitalis glycosides. The most striking example is that of ouabain; the lethal dose by the cardiac action in the rat is between 100 and 200 times that for the

cat. Ouabain has relatively little convulsant action, but the high dosage necessary in the rat applies also to the most convulsant of the materials, amorphous scilliroside; here the LD50 by the cardiac action in the rat is about 20 times the lethal dose for the cat (cardiac action), but even the LD50 by the convulsant action in the rat is 10 times the lethal dose for the cat. Therefore, even if the susceptibility of the central nervous system to scilliroside were the same in the cat and rat, the cat would fail to show convulsions because the cardiac action would prove fatal before there was sufficient time for the development of the action on the central nervous system.

From the standpoint of the use of the red squill material for its cardiac action in man, it may be noted that the full digitalizing intravenous dose in man is about 0.05 cat unit per kg. as against approximately 2 cat units per kg. as the minimal convulsant dose in rats, or approximately 40 times the therapeutic dose.

SUMMARY AND CONCLUSIONS

1. A study was made of the convulsant and cardiac actions of amorphous scilliroside (a highly purified material of red squill), urginin (red squill), scillicin (white squill), and several other principles of the digitalis group obtained from digitalis purpurea, digitalis lanata, oleander, convallaria, and strophanthus.

2. Contrary to the prevailing opinion, the conspicuous convulsant action of red squill in the rat is not only a peculiarity of the drug but also of the rat.

3. Evidence is presented which strongly suggests that the convulsant action of red squill in the rat is not due to a special convulsant principle, but is a function of its cardiac glycosides (or their genins).

4. Amorphous scilliroside causes poisoning in the rat by a slow convulsant action on the central nervous system after small doses, and by a rapid cardiac action without convulsions after large doses.

5. The white squill glycoside, scillicin, is less potent than scilliroside in the rat. It has highly convulsant properties in the rat, but there is the possibility that its convulsant action might be due to admixture with red squill glycoside.

6. Amorphous scilliroside acts like a typical digitalis glycoside in the frog, cat, and man.

7. The digitalis glycosides produce convulsions in the rat (digitoxin, gitalin, folinerin, ouabain). The convulsant action appears to be more highly developed in some (digitoxin) than in other (ouabain) members of the group.

8. There is indication that differences in the convulsant activity of different members of the digitalis group are due, in part, to differences in fixation or the speed of elimination, and in part, to differences in the convulsant factor in the molecular structure of the glycosides.

9. The convulsant response of the rat provides a means for qualitative differentiation of the glycosides of the digitalis group.

same regularity as in the case of scilliroside. Scillicin, a water-soluble glycosidal material from white squill regularly caused convulsions by intramuscular injection in the rat, and death by the cardiac action after a dose about 5 times as large. However, the white squill material failed to cause convulsions after intravenous doses; in these cases the rat either recovered or died of the cardiac action within a few minutes. The evidence leaves open the possibility that the convulsant actions of scillicin may be due to admixture of small amounts of red squill. In the case of ouabain and folinerin, it was found that while single doses failed to cause convulsions, repeated doses often brought on a convulsant state. One of the effects of repeated doses was to produce a sustained period of poisoning allowing for the development of the convulsant action which requires several hours.

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THE TOXIC ACTION OF PREPARATIONS CONTAINING THE OXYGEN-LABILE HEMOLYSIN OF STREPTOCOCCUS PYOGENES

IV. COMPARISON OF CARDIOTOXIC ACTION WITH THAT OF SAPONIN[1]

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The hemolysin-containing fraction of the supernatant fluid of *Streptococcus pyogenes* cultures is capable of producing a fatal toxemia in mice (1, 2). It has been shown (3) that intravenous injection of a sublethal quantity of the hemolysin-containing fraction induces in mice a state of refractoriness to a lethal dose. The evidence presented (3) indicates that the refractoriness does not depend upon the formation of antibody, but upon a different mechanism, the nature of which is not known. Investigation of the specificity of the protective effect showed that mice which are refractory to the streptococcal preparation also exhibit decreased susceptibility to the toxic action of saponin. In addition, it was demonstrated that mice injected with a sublethal quantity of saponin become refractory to saponin, and that these mice are refractory also to the streptococcal preparation. The fact that both saponin and the streptococcal preparation induce in mice a state of decreased susceptibility to the lethal effect of both agents suggests that there may be a fundamental similarity in the mechanism of action of the two toxic agents. In order to acquire further information concerning the apparent similarity of action of the two agents, it is desirable to compare the effects of saponin with those of the bacterial preparation under physiologically less complex conditions than are presented by intact animals. It has been found (4) that the isolated frog's heart responds in a characteristic and predictable manner when it is exposed to suitable concentrations of the streptococcal preparation. Since the frog's heart is sensitive also to saponin (5, 6), it was decided to utilize the isolated frog's heart as a test organ and to compare the effect of saponin with that of the bacterial product.

METHODS AND MATERIALS. The hemolysin-containing fraction of the supernatant fluid of *Streptococcus pyogenes* cultures was prepared in the manner described previously (4)[2]. The toxic preparation obtained in this way is designated SPA (streptococcal preparation A).

The toxic activity of the streptococcal preparation is oxygen-labile, therefore, activation

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be directed specifically against SPA. In order to see if the *liberation* of inhibitor is specifically associated with SPA, the following experiment was performed. Each of several hearts was perfused with a solution of saponin containing 0.2 mgm. per cc. When systolic standstill had occurred the solutions were removed from the hearts, pooled, and labeled "Solution C." In order to test Solution C for inhibitory activity it was necessary first to remove the residual saponin. This was accomplished by perfusing several hearts with Solution C, a process which causes saponin to become fixed to the heart tissues (8) thereby removing it from solution. After this treatment Solution C, which no longer possessed contracturing activity, was tested for inhibitory activity against a contracturing dose of SPA. No inhibitory activity was detected. It is evident that saponin unlike SPA, does not cause the heart to liberate an SPA-inhibitor.

Sensitization of heart to SPA by preliminary treatment with saponin. Although the results of the foregoing experiments fail to indicate a similarity in the actions of saponin and SPA, a further comparison of the two agents can be made. What, for example, would be the effect of a sub-threshold dose of saponin on the susceptibility of the heart to SPA? In order to answer this question a heart was exposed to a dose of saponin which produced no visible effect. This dose was 1 cc. of Ringer's solution containing 0.067 mgm. saponin. After 5 minutes the heart was washed with two portions of Ringer's solution and then exposed to SPA 1:80. The heart underwent almost complete systolic contracture, (Fig. 1 A). In a normal control heart, SPA 1:80 in a single dose, induced neither contracture nor any other visible effect.

Sensitization of heart to saponin by preliminary treatment with SPA. In order to find out whether SPA increases the susceptibility of the heart to saponin, a heart was exposed to SPA 1:80. There was no obvious effect. After five minutes the heart was washed with two portions of Ringer's solution, and then exposed to a solution containing 0.05 mgm. saponin per cc. The heart slowly developed systolic contracture, attaining standstill in about eight minutes (Fig. 1 B). In a normal control heart, a solution containing 0.05 mgm. saponin per cc. failed to induce contracture. The results of this experiment and the preceding one show that exposure of the heart to a subthreshold dose of saponin causes the heart to become more sensitive to SPA, and conversely, that a dose of SPA which does not produce an obvious effect causes the heart to become more susceptible to saponin.

Reinforcing action of SPA and saponin. The results of the preceding experiments suggest that a solution containing both saponin and SPA in subthreshold amounts may produce an effect which is not produced when either agent is tested separately. A solution containing saponin, 0.067 mgm. per cc., and SPA, 1:80, was introduced into a normal heart. Systolic standstill was induced almost instantly (Fig. 1 C) although neither agent by itself induced contracture. The results of this experiment and those described above show that a subthreshold dose of either agent causes the heart to become more sensitive to the other agent, and also that the two agents, when acting simultaneously, actually reinforce each other.

with a reducing agent is necessary. SPA at pH 7.4 was activated by mixing with 0.1 per cent cysteine and placing for 10 minutes in the refrigerator. The activated SPA was diluted with Ringer's solution before applying it to the frog's heart.

The saponin used was a commercial preparation (Merck) of undefined purity. A one per cent solution of saponin was prepared each day and diluted with Ringer's solution to the proper concentration prior to application to the heart.

Most of the experiments were carried out in the months of October, November and December. Isolated hearts of *Rana pipiens* were prepared according to Straub's technique (7). The perfusion solution had the following composition: NaCl 0.65 per cent, KCl 0.01 per cent, CaCl₂ (anhydrous) 0.02 per cent, NaHCO₃ 0 005 per cent. Oxygenation was maintained throughout.

EXPERIMENTAL. *Failure of saponin to induce homologous sensitization.*[3] SPA, as described earlier (4) induces systolic contracture of the isolated frog's heart. The cardiotoxic action is not a simple one because *two* administrations of SPA are required for the development of contracture. The requirement for two administrations does not depend upon a cumulative action but upon the fact that the first administration of SPA changes the state of reactivity of the heart tissues and induces a type of sensitization to the second application of SPA. The sensitization is a characteristic and apparently unique feature of the action of SPA.

There arises the question of whether a small dose of saponin can sensitize a heart to a second dose of saponin. The smallest quantity of saponin which regularly produced systolic contracture was determined with several hearts to be 0.13 mgm. Since saponin is known to have a cumulative action, it was necessary to choose a dose, two applications of which amount to less than 0 13 mgm. A dose of 0.05 mgm. was applied to a normal heart, and after five minutes, the heart was washed twice with Ringer's solution. A second application of the same dose of saponin produced no effect. The result shows that saponin, unlike SPA, fails to sensitize the heart to a second application of saponin.

Failure of SPA inhibitor to neutralize action of saponin. Another feature of the cardiotoxic action of SPA is that a single application causes the heart to release into the Ringer's solution which bathes the heart, a protective substance or "inhibitor" which is capable of preventing the contracture-producing action of SPA (2) The Ringer's solution containing inhibitor may, for convenience, be called "inhibitor fluid."[4] In order to find out whether inhibitor fluid prevents the contracture-producing action of saponin as well as that of SPA, 0.2 mgm. saponin dissolved in 1 cc. of inhibitor fluid was applied to a normal heart. Within four minutes the heart underwent systolic contracture. It is evident that the inhibitor failed to prevent the contracture-producing action of saponin.

Failure of saponin to cause release of an inhibitor active against SPA. The results of the previous experiment suggest that the activity of the inhibitor may

[3] This experiment and those which follow have been performed two or more times.

[4] Using the isolated frog's heart a solution can be tested for inhibitory activity by determining whether it will completely prevent the development of systolic contracture produced by the second application of SPA. Estimation of the potency of inhibitor fluid shows that the fluid can be diluted four or five times and still show inhibitory activity.

contracture of the isolated frog's heart. (b) SPA is lethal upon intravenous injection into mice. (c) SPA lyses erythrocytes *in vitro*.

The inhibitor from the frog's heart, even when diluted, is capable of preventing the development of systolic contracture of the frog's heart, and furthermore, it is known (2) to be capable of preventing the lethal effect in mice. In addition, the effect of the inhibitor upon hemolysis has been studied. It has been found that inhibitor fluid, taken directly from a heart which has been exposed for the first time to SPA, itself possessed hemolytic activity for washed, human erythrocytes at 37° C. The hemolytic activity of the inhibitor fluid was found to be

TABLE 1

Hemolysis induced by SPA alone, by saponin alone, and by both together

FINAL CONCENTRATION OF LYSIN		PER CENT HEMOLYSIS AFTER				
SPA	Saponin	5'	15'	30'	60'	120'
1:20,000	None	0	10	85	100	100
1:20,000	1:40,000	0	tr	40	65	90
1:20,000	1:80,000	0	0	0	tr	100
1:20,000	1:160,000	0	0	0	0	100
1:40,000	None	0	0	0	0	0
1:40,000	1:40,000	0	tr	30	65	80
1:40,000	1:80,000	0	0	0	0	0
1:40,000	1:160,000	0	0	0	0	0
1:80,000	None	0	0	0	0	0
1:80,000	1:40,000	0	tr	40	60	80
1:80,000	1:80,000	0	0	0	0	0
1:80,000	1:160,000	0	0	0	0	0
None	1:40,000	0	tr?	25	50	80
None	1:80,000	0	0	0	0	0
None	1:160,000	0	0	0	0	0

tr = trace.

neutralized by the addition of a small quantity of horse serum containing anti-streptolysin O and by cholesterol, and appears therefore, to represent residual hemolytic activity of SPA. Inhibitor fluid after ten minutes in a boiling water bath no longer retained its hemolytic activity. Heat-treated inhibitor fluid was still capable of preventing the contracture-producing and the lethal actions of SPA, but failed to affect the rate of hemolysis induced by a small quantity of fresh SPA. These findings show that the inhibitor fails to prevent the hemolytic action of SPA.

Failure of inhibitor fluid to prevent the sensitizing action of SPA on the frog's heart. As shown by the foregoing experiments, inhibitor fluid, although unable to inhibit the hemolytic action of SPA, is capable of preventing the *lethal* effect

Absence of additive effect of combinations of SPA and saponin on hemolysis.
The results of the preceding experiments as well as those presented elsewhere
(3) show that a certain degree of parallelism exists in the pharmacological effects
produced by saponin and those produced by SPA. In view of this finding, and
in view of the fact that both agents are strongly hemolytic, experiments were
undertaken in order to determine whether the hemolytic actions of saponin and
SPA reinforce each other.

The method of studying hemolysis has been described elsewhere (9). Con-
centrations of saponin and SPA were chosen with a view to finding out whether
the hemolytic actions of the two agents are additive. Ten cc. of a suspension of
washed, human erythrocytes, 0.7 per cent by volume, were added to each of 15
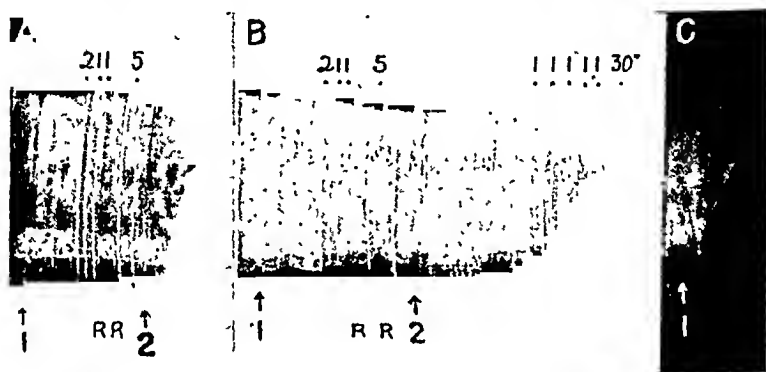
FIG. 1 SYNERGISM OF CARDIOTOXIC EFFECTS OF SPA AND SAPONIN ON THE
ISOLATED FROG'S HEART (STRAUB). RINGER'S SOLUTION 1 0 CC. KYMO-
GRAPHIC RECORDS OF AMPLITUDE OF VENTRICULAR CONTRACTION

A· At arrow 1, Saponin 0.067 mgm.; At arrow 2, SPA 1:80 in Cysteine 1:2500. B: At
arrow 1, SPA 1:80 in cysteine 1:2500; At arrow 2, Saponin 0 050 mgm   C At arrow 1,
SPA 1:80 in cysteine 1:2500 containing 0 067 mgm. saponin. R. Washing with Ringer's
solution. Figures indicate in minutes the period of arrest of the drum

tubes containing various concentrations of either SPA or saponin or both. The
mixtures were incubated at 20° C., with stirring at intervals. After 5, 15, 30, 60
and 120 minutes, 1 cc. volumes were withdrawn, centrifuged, and the degree
of hemolysis estimated by comparison with standard solutions of hemoglobin.
The course of hemolysis is indicated by the data of table 1. It can be seen that
not only are the hemolytic actions of the two agents not additive, but that SPA
hemolysis is temporarily inhibited by sub-hemolytic concentrations of saponin.
This finding contrasts with those of preceding experiments in which it was found
that the cardiotoxic actions of saponin and SPA reinforced each other.

Failure of inhibitor fluid from frog hearts to prevent hemolysis by SPA. The
toxic properties of the streptococcal preparation can be demonstrated in at least
three biological systems: (a) Under appropriate conditions SPA produces systolic

DISCUSSION. Formulation of a concept relating the cardiotoxic action of saponin to that of SPA has presented some difficulty. It is desirable to explain how the two agents produce sensitization by processes which are evidently similar but certainly not identical. It is possible to put forth tentatively a hypothesis which will assist in the interpretation of our results:

It may be considered that in the normal heart two barriers, X and I, cover a receptor, R, which is the ultimate site of action of a cardiotoxic agent. In order for the receptor to become accessible to a contracture-producing agent, it is necessary that either one of the two barriers, X or I, first be removed. SPA accomplishes this by freeing I (inhibitor); any further action, under the particular conditions offered by the Straub heart, is prevented by the antagonistic effect of I upon SPA. When the heart is washed with Ringer's solution, I is removed, and a second application of SPA can then reach R and produce systolic contracture.

The mechanism by which saponin uncovers the ultimate site of action, R, is not known. Since saponin does not cause liberation of an SPA-inhibitor when it is applied to the heart, one can postulate that exposure of R is brought about not by removing I, but by displacing another barrier, X. When this occurs, R becomes accessible to cardiotoxic agents. Subthreshold amounts of saponin are known (10) to cause an increased susceptibility of the isolated frog's heart to digitoxin. This effect has been attributed to an alteration in permeability brought about by combination of saponin with the cholesterol in the membranes of the heart cells (11). A similar interpretation has been given to the observation that saponin, in amounts not sufficient·for the induction of systolic contracture of the isolated frog's heart, will shorten the latent period which precedes the cardiotoxic action of digitalis glycosides.

Attention may be drawn to the observation that the *sensitizing* and *contracturing* actions of SPA are completely independent. This interesting situation can be accounted for by assuming that the active component of SPA possesses two reactive groups: one sensitizes the heart; the other produces contracture. The concept of the twofold action of SPA is supported by the observations that the sensitizing action of SPA is not prevented by the inhibitor while the contracturing action is. It is notable also that the hemolytic activity of SPA is not neutralized by the inhibitor. In its failure to be prevented by the inhibitor, the hemolytic activity resembles the sensitizing rather than the contracture-producing action of SPA.

In conclusion, it is clear that the cardiotoxic action of saponin and that of SPA are not identical. The most obvious difference is the release of inhibitor, an occurrence which always accompanies exposure of a normal heart to SPA, but which appears never to accompany exposure of a heart to saponin. In spite of this difference, the fact that either agent renders a heart more susceptible to the other, and the fact that both agents acting simultaneously exhibit an additive if not synergistic action, show that a single mechanism may underlie the cardiotoxic action of the two agents.

of SPA in mice as well as the *contracturing* action of SPA in the isolated frog's heart. Whether the inhibitor fluid can inhibit the *sensitizing* action as well as the contracturing action of SPA is not known. The purpose of the present experiment was to determine whether inhibitor fluid is capable of inhibiting sensitization of the frog's heart by SPA.

SPA, 1:50, was diluted with an equal volume of inhibitor fluid, and then applied to a normal heart. After five minutes, the heart was washed twice with Ringer's solution. SPA, 1:100 was now applied to the heart. Systolic contracture developed within two minutes. From these results it may be inferred that the inhibitor does not prevent the sensitizing action of SPA.

Release of inhibitor from heart treated with saponin and subsequently with SPA. The normal heart, as has been repeatedly emphasized, does not undergo systolic contracture when exposed to a single application of diluted SPA. There are, however, at least two ways in which the state of reactivity of the heart can be modified in such a way that it will undergo systolic contracture in response to SPA; (a) By the application of diluted SPA followed by washing the heart with Ringer's solution; (b) by the application of a subthreshold dose of saponin. The manner in which SPA alters the reactivity of the heart has been shown (2) to involve the release and removal from the heart tissues of a protective substance or inhibitor. On the other hand, the manner in which saponin alters the reactivity of the heart is unknown. This must differ, however, from that of SPA since the action of saponin, as has been described above, is not accompanied by the release of inhibitor. It was of interest to find out whether a heart treated with a subthreshold dose of saponin would release inhibitor when subsequently exposed to SPA. For this experiment two hearts are used: Heart A and Heart B.

HEART A. Solution containing 0.067 mgm. saponin per cc. applied: *no effect.*
After five minutes, heart washed twice with Ringer's solution.
Solution of SPA 1:70 applied: *systolic standstill.*
After five minutes, fluid removed from heart and designated "A".
HEART B. Solution containing SPA 1:70 applied: *no effect.*
After five minutes, heart washed twice with Ringer's solution. Solution containing SPA 1:35 plus an equal volume of "A" applied: *no effect.*
After five minutes, heart washed twice with Ringer's solution.
Solution containing SPA 1:70 applied: *systolic standstill.*

The results of this experiment indicate that a heart whose reactivity to SPA has been modified by application of a subthreshold dose of saponin releases inhibitor in response to a subsequent application of SPA. It is notable that Heart A not only released inhibitor but also underwent systolic standstill. In previous experiments (2) it was found that fluid removed from hearts which had undergone systolic contracture in response to the second (or "contracturing") dose of SPA did not possess inhibitory activity. The results of the present experiment permit a further interpretation of this earlier finding. It is evident that the release of inhibitor is correlated neither with the failure of the heart to undergo contracture nor with the development of contracture, but rather with the first contact of the heart with SPA.

METABOLIC ACTION OF THE CARDIAC GLYCOSIDES

I. Influence on Respiration of Heart Muscle and Brain Cortex[1]

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The search for an explanation of the mode of action of the cardiac glycosides has led a number of authors to examine the influence of this important group of cardiac drugs on the energy-yielding processes in the heart. The great majority of these investigations has dealt with effects on the energy metabolism, particularly the respiration, of the heart *in vivo*. Only few attempts have been made to relate the cardiotonic and cardiotoxic properties of the cardiac glycosides to an ability of producing changes in the respiratory activity of heart muscle *in vitro*, i.e. changes independent of those in mechanical activity. Frühauf (1) reported that the cardiac glycoside strophanthin inhibited the oxygen uptake of minced mammalian heart muscle. Salomon and Riesser (2), who repeated some of Frühauf's experiments, failed to observe any effect of low or high concentrations of strophanthin and digitoxin. Similar experiments of Genuit and Haarmann (3) likewise yielded negative results. All these studies are open to criticism, because without exception the rates of oxygen consumption were abnormally low.

Recently, Lévy and her collaborators (4-6) and this author[1] reported simultaneously a characteristic effect of cardiac glycosides on actively respiring heart muscle slices, consisting in an increase and, at sufficiently high concentrations, a depression of respiration. In the present study, the respiratory activity of brain cortex slices is shown to be similarly affected, and the nature of the influence of the cardiac glycosides on the respiration of heart muscle and brain cortex is explored.

METHODS. Hearts and brains of guinea pigs were used in most experiments. Other guinea pig tissues were used for comparison. The animals were healthy young adult males and non-pregnant females weighing 650 to 800 grams. They were killed with a blow on the neck or by decapitation. Heart, cerebrum, or other organs were removed immediately and placed in a beaker filled with an isotonic salt solution to be described below, cooled to about 15°C. The heart was allowed to beat a few times and thus have its cavities washed free of blood. Slices of left ventricular muscle, cerebral cortex, or other tissue, about 0.4 to 0.5 mm. thick, were cut free hand and placed in a shallow beaker containing 8 cc. of the isotonic solution, kept agitated by a stream of oxygen bubbles. The slices were then transferred to Warburg vessels containing 2.85 to 2.92 cc. of this medium. Usually about 150 mg. of ventricular or 80 mg. of cerebral slices were used per vessel. The composition of the medium was as follows: 0.093 M NaCl, 0.0048 M KCl, 0.0013 M CaCl$_2$, 0.0012 M

SUMMARY

1. The cardiotoxic action of saponin has been compared with that of p tions containing the oxygen-labile hemolysin of streptococci.

2. Exposure of the heart to a single application of the streptococcal p tion (SPA) increases the responsiveness of the heart to saponin. Con exposure of a heart to a subcontracturing dose of saponin increases its resp ness to SPA.

3. Exposure of the heart to a mixture of the two agents produces an systolic contracture, which is not elicited by either agent acting alone in th concentration.

4. Exposure of the heart to a single application of SPA sensitizes the h a second application of SPA. Exposure of a heart to a comparable concen of saponin *fails* to sensitize to a second application of saponin.

5. The hemolytic actions of SPA and saponin are not additive.

6. Release of an inhibitor accompanies the first exposure of a heart t but does not accompany exposure of a heart to saponin in any of the con tions tested.

7. Inhibitor-fluid prevents the contracturing action of SPA, but p neither the contracturing action of saponin nor the sensitizing action o

8. It is postulated that the ultimate site of action of the cardiotoxic ag normally covered by two barriers, one of which can be displaced by sapon other by SPA, and that exposure of the ultimate site of action must occur either agent can produce systolic contracture.

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Libert (4) who reported that ouabain in high concentrations does not increase the rate of respiration of cardiac slices, but depresses it from the beginning. Since these investigators, however, took readings only once an hour, it is quite possible that the strong, but short-lasting initial stimulating action of high concentrations of the drug may have escaped their attention.

Tissue Specificity. Among the tissues listed in table 1, brain cortex alone resembles cardiac muscle in its response to ouabain. The respiration of brain cortex slices, like that of cardiac slices, can first be stimulated and then be inhibited by appropriate amounts of the cardiac glycoside. Considerably higher
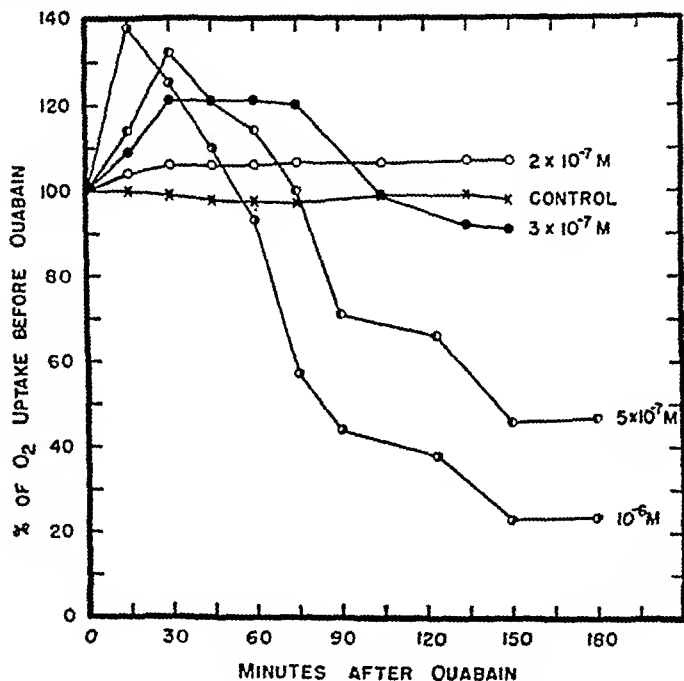
FIG. 1 EFFECT OF OUABAIN ON THE RESPIRATION OF SLICES OF GUINEA PIG HEART MUSCLE

concentrations, however, are required to bring about degrees of stimulation and and inhibition comparable to those produced in heart muscle. On the whole, the sensitivity of brain cortex to ouabain is approximately $\frac{1}{3}$ that of cardiac muscle. The intensity of action of ouabain on brain cortex was not materially altered by raising the ratio of tissue weight to medium volume to 1:20, i.e. the ratio employed with cardiac tissue. A nearly 100 per cent inhibition of brain respiration was produced by ouabain in concentrations 2×10^{-5} M and higher.

The lower sensitivity of brain cortex to ouabain and, presumably, other cardiac glycosides may have been responsible for the fact that Libert (5) failed to note

MgSO$_4$, 0.033 M sodium phosphate buffer of pH 7.4. It usually contained substrate, in most experiments 0.011 M (0.2%) glucose. The conventional Warburg technique was employed for the direct manometric measurement of oxygen consumption. The gas phase was oxygen, the temperature 38°C.

Following gas and thermal equilibration all tissue slices except those from cardiac muscle maintained a steady or fairly steady rate of respiration in the presence of glucose. The oxygen uptake of cardiac slices, however, declined during the first half hour by about 33 per cent. From then on it remained constant for 4 hours. The data given for cardiac slices do not include readings taken during the initial decline in the rate of oxygen uptake. Readings were taken every 15 to 30 minutes for approximately 4 hours. Runs were made in duplicates, whenever feasible, and all experiments were repeated at least twice with tissue from different animals. Part of the results are given as ml. oxygen consumed per mg. final dry weight of tissue per hour (Q_{O_2}). The final dry weight of the cardiac muscle slices was on the average 83 per cent that of the initial dry weight, the latter being taken after the preliminary bathing in oxygenated medium. The corresponding figure for the brain cortex slices was 94 per cent. The loss in dry weight (and also in wet weight) was not affected by ouabain, the cardiac glycoside used in this study. Ouabain, dissolved in not more than 0 3 cc , was added from the sidearm of the vessels following determination of the rate of oxygen uptake without the drug, i.e. usually after the 60 minute reading in the experiments with heart slices, and after the 30 minute reading in all other experiments. In this way, each vessel served as its own control. In addition, one or two overall controls, containing no ouabain, were run in each experiment.

Ouabain (g-strophanthin) was the cardiac glycoside of choice because of its water solubility. USP XII standard ouabain was used. It contains 4 molecules of water of crystallization, or 11 per cent water by weight. The concentrations are expressed in moles per liter. 10^{-6} moles/liter = 1:1,520,000 by weight. A 10^{-4} M stock solution in suspension fluid was prepared freshly each day.

RESULTS. *Magnitude and Character of the Effect.* The action of ouabain on the respiration of cardiac muscle slices incubated in glucose-containing medium is illustrated in figure 1. Concentrations as low as 2 and 3 $\times$ 10^{-7} M (1:7,600,000 and 1:5,000,000) produce an increase in the rate of oxygen consumption. At concentrations 3 $\times$ 10^{-7} M and higher, the action of ouabain becomes diphasic, i.e. the increase in respiration is followed by a depression. Both rate and extent of the rise and fall in respiration depend, up to a certain limit, on the concentration of the cardiac glycoside. 2 $\times$ 10^{-7} M is the threshold concentration for producing a noticeable effect. There is a slight, but persisting elevation in the rate of oxygen uptake above the control level. This difference falls within the range of experimental error, but was noted consistently. 10^{-6} M is the concentration at which nearly maximal effects are obtained. Following exposure to 10^{-6} M ouabain, oxygen consumption increases sharply to a peak of about 40 per cent above the control level. This increase is followed by a rapid fall. 1½ hours later, the respiratory activity has fallen to a level almost 80 per cent below normal. Concentrations of ouabain higher than 10^{-6} M were found to cause only a slightly greater initial increase in respiration and no significantly greater depression (cf. table 1). The respiratory quotient (R.Q.) of the cardiac slices was not changed significantly by ouabain and either remained constant at values ranging from 0.91 to 0.98 or increased slightly within these limits.

These findings are not wholly in agreement with those of Lévy, Schwob and

METABOLIC ACTION OF THE CARDIAC GLYCOSIDES. I

In brain, neither the intensity nor the time course of the ouabain effect is altered in the least when pyruvate or lactate is substituted for glucose. In all instances, 4×10^{-6} M ouabain produces an initial elevation in respiration of 30 per cent and a final depression of 70 per cent (cf. table 5 for comparison). In the presence of succinate, on the other hand, oxygen consumption is affected only to a moderate extent by 4×10^{-6} M ouabain. As in the case of heart muscle respiring in pyruvate, an initial increase does not take place, and the respiratory rate begins to fall below that of the controls after a period of 20 minutes. Taking the decline in the control QO_2 into account, this inhibition does not amount to more than 30 per cent.

To test the action of ouabain on heart muscle slices in the presence of succinate,

TABLE 2

Influence of ouabain on the respiration of slices of heart muscle and brain cortex in the presence of various substrates

Results are given in cmm. O_2 consumed per mg. final dry weight of tissue per hour.

MINUTES AFTER OUABAIN	HEART MUSCLE						BRAIN CORTEX					
	Pyruvate, 0.1%		Lactate, 0.1%		Glucose, 0.2%		Pyruvate 0.1%		Lactate, 0.1%		Succinate, 0.1%	
	Control	Ouabain 10^{-4} M	Control	Ouabain 10^{-4} M	Control	Ouabain 10^{-4} M	Control	Ouabain 4×10^{-4} M	Control	Ouabain 4×10^{-4} M	Control	Ouabain 4×10^{-4} M
—	7.2	7.3	5.0	5.3	5.5	5.2	12.9	12.9	11.1	11.4	8.2	8.5
7–22	7.0	6.7	4.9	7.2	5.5	7.2	13.0	16.8	11.0	14.4	7.5	7.8
22–37	6.7	4.2	5.1	6.5	5.6	6.3	12.9	15.1	11.1	11.6	6.4	5.0
127–142	4.7	1.7	3.3	1.0	5.2	1.4	12.4	3.8	9.2	2.2	3.0	2.1
144					Succinate, 0.1%							
149–154					28.3	27.1						
154–172					22.9	20.0						
247–262					5.8	1.7						

inhibition of respiration was produced first in glucose medium and succinate added next. This arrangement was necessary because the oxygen uptake of heart slices incubated in succinate declines very rapidly from initially high values, thus making it difficult to evaluate the effect of adding ouabain to this medium. Table 2 shows that succinate remains oxidizable in ouabain-poisoned heart muscle. Even in a concentration which is highly inhibitory to the respiration of heart muscle incubated in glucose, lactate, or pyruvate, the glycoside is unable to prevent the sharp increase in oxygen uptake caused by succinate. The oxygen uptake, depressed to over 70 per cent below normal by 10^{-6} M ouabain, increased nearly 20 fold immediately upon addition of succinate and approximately equalled that of the control slices. Thus the inhibitory action of ouabain on cardiac respiration cannot be due to damage of the succinic dehydrogenase or the cytochrome-cytochrome oxidase system.

Dependence of Respiratory Stimulation on Extracellular Substrate. Slices of

any action of digitoxin on brain respiration. A moderate inhibition of the respiration of guinea pig brain cortex by strophanthin has been reported by Weese and Wiegand (7). However, in contrast to the present findings, large concentrations (0.01 to 0.1 per cent) were required.

Table 1 shows that the rate of respiration of various tissues other than heart and brain cannot be augmented even by fairly high concentrations of ouabain. However, a slight or moderate inhibition occurs as a rule. In the presence of 10^{-5} M ouabain, the respiration of slices and other forms of intact tissue of lung, skeletal muscle, and kidney cortex is decreased by about 33 per cent, that of liver by 23 per cent, and that of spleen and testis by only about 10 per cent. Higher concentrations of ouabain were not used with these tissues.

Substrate Specificity. The response of heart muscle and brain cortex slices to

TABLE 1

Response of various tissues of the guinea pig to ouabain

The results are expressed as per cent initial increase (+) and as per cent final depression (−) of the rate of oxygen uptake before addition of ouabain.

TISSUE	Q_{O_2}	CONCENTRATION OF OUABAIN					
		5×10^{-7} M		2×10^{-6} M		10^{-5} M	
Heart	−5.3	+33	−55	+41	−79	+48	−81
Brain cortex...	−12.9	+ 0	−10	+16	−50	+36	−92
Lung	−7.0	+ 0	− 9	+ 0	−18	+ 0	−35
Kidney cortex	−16.8	+ 0	− 7	+ 0	−16	+ 0	−33
Skeletal muscle*..	−2.3	+ 3	− 2	+ 0	− 7	+ 0	−32
Liver	−4.6	+ 0	−14	+ 0	−19	+ 0	−23
Testis..	−8.4	−	−	−	−	+ 0	−12
Spleen...	−7.8	−	−	−	−	+ 1	−10

* Strips of m. gastrocnemius.

ouabain was studied further in the presence of some non-carbohydrate substrates known to be readily oxidized by these tissues. These substrates were pyruvic acid, lactic acid and succinic acid. The sodium salts were used, in a concentration of 0.1 per cent (as the anions).

The results of these experiments are presented in table 2. It is seen that the oxygen uptake of heart muscle slices incubated in lactate medium is first stimulated and subsequently depressed by ouabain to the same extent as in glucose. In pyruvate medium, on the other hand, the initial increase in respiratory activity is lacking. Oxygen consumption, which initially is as high as following maximal stimulation by ouabain in glucose or lactate medium, decreases sharply, after a latent period of 20 minutes, upon addition of the cardiac glycoside. A final depression of 77 per cent, or 70 per cent when corrected for the changes in the controls, was obtained after 90 minutes. This depression is of the same order as that obtained in glucose and in lactate medium, but occurs in a shorter time.[2]

[2] Repetition of these experiments with sodium pyruvate prepared and kindly supplied by Dr. F. Lipmann gave the same results.

The R.Q. of the cardiac slices respiring in substrate-free medium was found to average 0.90 during the first 2 hours of incubation and thus did not differ greatly from the R.Q. obtained in glucose medium.

Irreversibility of Respiratory Inhibition and Effect of Repetitive Addition. In order to determine whether the effects produced by ouabain persist in ouabain-free solution, brain cortex slices exposed to the drug were washed and transferred to vessels containing fresh, ouabain-free medium. The transfer was later repeated or followed by a second treatment with ouabain. This technique was not employed with heart muscle slices, since this tissue does not withstand transfer

TABLE 4

Effects of washing and of a second exposure to ouabain on the respiration of ouabain-treated brain cortex slices

Transfer of the slices was effected, following washing, into ouabain-free medium. The results are expressed as per cent of the initial rate of oxygen uptake.

TIME AFTER OUABAIN	4 × 10⁻⁶ M OUABAIN				CONTROL
	1	2	3	4	5
min.					
—	100	100	100	100	100
5–20	132	127	130	133	100
20–40	Transfer	Transfer	117	121	Transfer
40–55	68	66	94	98	98
55–70	69	65	74	76	97
70–80	68	Ouabain*	Ouabain†	67	98
80–95	68	78	48	57	100
95–110	69	66	36	44	98
110–125	70	50	28	36	101
125–140	68	30	21	32	99
140–155	69	20	18	30	98
155–185	Transfer	16	14	Transfer	Transfer
185–200	66	16	12	28	94
200–215	64	15	12	27	92

* 4 × 10⁻⁸ M.
† 8.8 γ, making the final concentration 8 × 10⁻⁸ M.

into fresh medium without a decline in respiratory activity. In contrast, the respiration of brain cortex slices, as the control data in table 4 show, was practically unimpaired following transfer.

Table 4 demonstrates that the inhibitory action of ouabain on brain respiration, like its toxic action on the heart *in vivo*, cannot be reversed by washing. The first transfer (columns 1 and 2) was made after 20 minutes of incubation with 4 × 10⁻⁶ M ouabain, at which time the initial increase in oxygen uptake had reached its peak. It is seen that the oxygen uptake in the new vessel not only failed to remain at the previous high level, but at once fell to nearly half this level. Comparison with columns 3 and 4 shows that during the first 15 minutes following transfer the slices respired at a considerably lower rate than if they had

myocardium incubated in nutrient-free medium respire, for a period of at least 2 hours, as actively or nearly as actively as in medium containing metabolites such as glucose which do not or do not markedly increase respiration. It was therefore possible to study the response of cardiac tissue to ouabain in the complete absence and in the presence of small amounts of exogenous substrate. Before the start of the experiments, the slices were washed twice for 5 minutes in 10 cc. oxygenated nutrient-free medium. Glucose was the substrate used. The concentration of ouabain was 5×10^{-7} M.

The results of these experiments (table 3) demonstrate, in agreement with similar data reported by Libert (8), that the stimulating action of ouabain on cardiac respiration is dependent upon the presence and concentration of appropriate substrate in the surrounding. In the absence of glucose, or of any substrate, respiration does not increase. As the concentration of glucose in the medium is

TABLE 3

Influence of exogenous substrate concentration on the effect of ouabain on respiration

Slices of guinea pig heart muscle, washed 2 x 5 min. in oxygenated substrate-free medium. Concentration of ouabain 5×10^{-7} M.

CONCENTRATION OF GLUCOSE	Q_{O_2}	PER CENT CHANGE OF OXYGEN UPTAKE AFTER ADDITION OF OUABAIN	
		Initial increase	Final depression
per cent			
0	−4.1	2	57
0.02	−4.4	14	50
0.05	−4.8	28	59
0.2	−4.6	35	56
1.0	−4 8	35	54

raised, stimulation of respiration becomes stronger. In 0.02 per cent glucose solution, the increase is 14 per cent. Maximal stimulation is obtained with 0.2 per cent glucose. A further increase in concentration is without effect.

It is also seen from table 3 that the inhibitory phase of the ouabain action is not dependent upon the presence or concentration of substrate in the environment. 5×10^{-7} M ouabain depresses oxygen consumption by about 55 per cent in the absence and at all concentrations of glucose tested. Since it was shown earlier that replacing glucose by lactate or pyruvate does not alter the intensity of inhibition, it follows that these metabolites are likewise without effect on the inhibitory power of ouabain.

The values of Q_{O_2} listed in table 3 are somewhat lower, even for cardiac slices respiring in glucose, than the values usually obtained in the present study (cf. tables 1 and 2). This diminution in respiratory activity was due undoubtedly to the additional washing to which these slices had been subjected. It is most likely that the decline in the oxygen uptake of cardiac slices, which as a rule occurred in all the present experiments during the first half hour of incubation, similarly constitutes a dilution phenomenon.

oxidation of lactate by homogenate of heart muscle of vitamin E deficient guinea-pigs. This effect, which also could be elicited by cholesterol, digitonin, and certain sex hormones, is apparently unrelated to the phenomena reported here.

The data in the last two columns of table 5 furnish another illustration of the inability of ouabain to influence the activity of cell-free respiratory systems. The glycoside was incubated with an isolated respiratory enzyme chain made up of preparations of glucose dehydrogenase (acetone liver extract) (12), containing substrate and coenzyme[2], and a cytochrome oxidase preparation containing cytochrome and flavoprotein. This system catalyzes the aerobic oxidation of glucose to gluconic acid(13). Table 3 shows that ouabain had no effect on the activity of this system, although its concentration was more than 1000 times that required to produce a 50 per cent inhibition of oxygen consumption in guinea pig

TABLE 5

Effect of ouabain on the oxygen uptake of homogenized guinea pig heart and brain and of an isolated respiratory enzyme system

3.0 cc. of 15% homogenates in M/30 phosphate buffer of pH 7.4 and 0.2% glucose.

Composition of respiratory enzyme system: glucose dehydrogenase preparation (equivalent of 1.8 gm. beef liver), 0.2 mg. 80% pure coenzyme I, cytochrome oxidase preparation (equivalent of 0.65 gm. pig heart), 4.8×10^{-6} M added cytochrome c; 0.13 M glucose; 0.033M phosphate buffer of pH 7.4. Total volume: 3.0 cc.

Air; 38°C.

TIME	HEART			BRAIN		RESPIRATORY SYSTEM	
	Control	Ouabain 5×10^{-7} M	Ouabain 10^{-4} M	Control	Ouabain 10^{-4} M	Control	Ouabain 5×10^{-4} M
min	cmm.	cmm.	cmm.	cmm.	cmm.	cmm.	cmm.
10	179	180	173	94	84	37	37
20	351	355	340	177	156	75	74
30	515	523	502	250	219	114	111
40	657	668	643	315	275	156	151
50	762	776	747	374	326	199	193
60	831	847	815	428	373	244	237

heart muscle slices. The catalytic activities of the dehydrogenase and of the cytochrome oxidase preparations, tested separately, were likewise unaffected. On the other hand, the sensitivity of the complete system to respiratory poisons such as chlorobutanol (cf. 14) was of the same order as that of guinea pig heart slices.

It can be concluded from the above experiments that ouabain does not directly interfere with the activity of respiratory catalysts, and that its influence on the respiration of heart muscle and brain cortex is dependent upon the integrity of cellular structure.

DISCUSSION. It was shown (table 3) that utilization of glucose and, by in-

[2] Diphosphopyridinenucleotide, obtained through the courtesy of Dr. J. A. Aeschlimann of Hoffmann-La Roche, Inc.,

remained in continuous contact with ouabain in the original medium.   This new
low rate was subsequently maintained without further change.   On the other
hand, when the slices were placed in fresh medium after the level of maximal
inhibition obtainable with 4×10^{-5} M ouabain was reached, no further depression
of significance was noted (column 4).   Repetition of the transfer procedure, per-
formed after 2 hours of shaking in ouabain-free solution, likewise failed to produce
a significant change in the rate of oxygen uptake (column 1).   There was no
further loss in activity, such as occurred following the first transfer, but neither
was there any gain.   The tissue, having once come in contact with ouabain,
remained irreversibly damaged.

When a second dose of ouabain was given after the slices had been transferred
into fresh medium and the new lowered level of activity had been established,
the familiar diphasic change took place (column 2).   Respiration was at first
increased by 20 per cent (in reference to the new level) and then depressed by 75
per cent.   On the other hand, a second addition of ouabain to slices which had
remained in the original, ouabain-containing solution did not stimulate respira-
tion, but at once accelerated the previously initiated decline in activity (column
3).

Role of Cell Structure.   If the responsiveness of heart muscle and brain cortex
slices to ouabain is linked to structural characteristics of the myocardial and
brain cortex cell, the respiratory action of the cardiac glycoside ought to be
observable only in intact and not in destroyed tissue.   To put this supposition
to test, suspensions of heart and brain tissue, which had been disintegrated in
the glass homogenizer of Potter and Elvehjem (9), were exposed to the drug.
The homogenization process was carried out for 8 minutes under ice cooling
in hypotonic (M/30) phosphate buffer and yielded a heart homogenate which
respired more vigorously than heart slices, while the resulting homogenate of
brain, in conformity with the findings of Elliot and Libet (10), was considerably
less active than brain cortex slices.   Ouabain was placed directly into the main
compartment of the reaction vessels.   The gas phase was air.   The results of
these experiments are presented in table 5.

It is seen that neither low nor high concentrations of the cardiac glycoside had
any significant influence on the oxygen uptake of the homogenate of heart muscle.
The respiration of the brain homogenate was depressed by 10 to 12 per cent
throughout the entire experiment.   This is a minor effect compared with the
nearly complete inhibition of respiration which can be produced by ouabain in
brain cortex slices.   It was due in all likelihood to the presence of intact or
partially intact cells in the suspension, and it may safely be assumed that an
effect on the homogenate proper was lacking.   This contention is supported by
the finding that the respiration of brain suspensions prepared by homogenizing
the tissue for 1 minute in warm isotonic solution (sodium chloride-phosphate-
glucose), thus minimizing cytolysis (10), was inhibited by ouabain to the extent
of 60 per cent.

Ouabain and digitoxin were recently reported (11) to increase the anaerobic

The question remains to be answered, in what way the phenomena described here may be related to the action of the cardiac glycosides *in vivo*. The existence of such a relation can hardly be in doubt, considering that the potency of the influence of ouabain on the respiration of cardiac slices is of the same order of magnitude as that of its systolic action. The minimal concentration of ouabain capable of producing systolic standstill of the isolated heart of young guinea pigs weighing 250 grams, perfused in a closed system with 15 volumes of buffered Tyrode solution at 33° C, has been determined by Lendle and Schwerbrock (17) to be 1:1,500,000 (10^{-6} M). Working under analogous conditions, i.e. with slices of heart muscle of 250 gram guinea pigs at a temperature of 33° C and at a tissue concentration of 1:15, the inhibition of respiration produced by 10^{-6} M ouabain was found to be 53 per cent. Such a severe impairment of the basal respiration can probably not be tolerated for any length of time by the heart, which is an organ known for its high susceptibility to damage by anoxia. Hence serious interference with normal respiratory function appears to be a likely cause of the toxic action of ouabain and other cardiac glycosides on the heart *in vivo*. Similarly, inhibition of respiration in brain cortex might be a cause of the disturbances in central nervous function observed in digitalis poisoning. Of significance in this connection may be the observation of Dearing *et al.*(18) that the anatomical lesions found in the myocardium and cerebral cortex of animals poisoned with digitalis are of the same type as those produced by prolonged deprivation of oxygen.

One may be tempted at first sight to extend the foregoing considerations and regard stimulation of respiration the equivalent of therapeutic action *in vivo*. Such interpretation may prove fallacious. Diphasic changes in the rate of respiration of the type produced by ouabain, and likewise believed to be associated with disturbances in cell permeability, have been observed in various tissues as a result of mechanical or chemical injury (19–21). By means of transferring brain cortex slices from ouabain-containing into ouabain-free medium (table 5) it was possible to show that at the time when respiratory activity had just reached its maximum, the biochemical lesion of ouabain poisoning was already well developed. This finding suggests that the initial increase in the respiration of ouabain-treated slices of brain cortex and heart muscle, far from being a "therapeutic" effect, may be an early sign of tissue damage.

It must be pointed out, however, that transfer experiments with non-inhibitory concentrations of ouabain were not performed. Lévy *et al.*(4) have reported that slices of cardiac muscle of rats which had received injections of non-toxic doses of ouabain, respired at a higher than normal rate, without subsequent inhibition of activity. Granted there may be a connection between stimulation of respiration and cardiotonic action, a causal relationship, such as was proposed to exist between inhibition of respiration and cardiotoxic action, appears most unlikely, for the reason alone that the cardiac glycosides are able to exert their full positive inotropic action in cases of myocardial insufficiency not caused by impairment of metabolic energy production (22). Instead, the increase in respiration pro-

ference, of lactate and perhaps other substrates added to the medium, but not
of substrate originally present inside the cell, is temporarily speeded up under the
influence of ouabain.   Since, according to the R.Q., carbohydrate is the pre-
dominant endogenous substrate of excised guinea pig heart muscle, it may be
inferred that this action is not or not exclusively brought about by increasing
the catalytic power or accessibility of the enzymes attacking glucose and its
intermediaries, but by making more exogenous substrate available to these
enzymes.   To accomplish this, the cardiac glycoside must facilitate the entry of
substrate into the cell.   Thus the present data lead to the conclusion that an
increase in the permeability of the cell surface to glucose, lactate, and probably
other oxidizable substrates is a likely basis for the stimulating action of ouabain
on the respiration of heart muscle and, by analogy, of brain cortex.   It follows
from these considerations that when availability of substrate is not a factor
limiting the respiratory rate, ouabain will be without stimulating action.   This
might be the explanation for the inability of the drug to increase the respiration
of cardiac slices in the presence of pyruvate

The observation (table 4) that inhibition of respiration can be precipitated
by placing ouabain-treated brain cortex slices into fresh medium can best be ex-
plained on the ground that one or more cellular constituents essential for main-
taining the normal rate of respiration diffused out of the cell and remained in
the old medium.   This explanation of the injurious effect of fresh medium on
ouabain-treated tissue implies that the inhibition by ouabain of the respiration
of slices of cerebral cortex and, by analogy, of cardiac muscle is essentially the
result of an abnormally high permeability of the cell to these diffusible oxidative
catalysts.   In agreement with this conclusion is the finding that the activity of
succinic oxidase, which functions without coenzyme and is intimately associated
with the insoluble fraction of the cell, is resistant to the inhibitory action of the
cardiac glycoside.   Thus it appears from the present data that both stimulation
and inhibition of respiration are consequences of the same disturbances in the
cell surface.   It may be recalled in this connection that the inotropic action of
the cardiac glycosides is thought (15) to be dependent, too, on their interaction
with constituents of the cell surface.

The inability of ouabain to suppress oxidation of succinate indicates also that
cytochrome c is not among the diffusible catalysts whose loss is held responsible
for the decline in oxygen uptake in the presence of glucose, pyruvate, or lactate.
Apparently the permeability changes produced by the drug are not drastic enough
to cause depletion of molecules of the size of cytochrome c.   The finding that
the total loss of solid cell constituents during long-lasting incubation, probably
mainly a loss of soluble proteins, is not greater in ouabain-poisoned than in
normal tissue, supports this contention   Against the occurrence of a general
breakdown of permeability barriers and of other gross changes in the cell surface
speaks also the fact that ouabain, according to estimations based on figures given
by Clark (16), is able to exert its full effect on the respiration of cardiac slices in
amounts capable of covering only a small fraction of the total cell surface area.

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duced by ouabain, and also the subsequent depression, may be regarded as manifestations of those changes, presumably in the cell surface, which induce the myocardial fiber to contract with greater force.

SUMMARY

1. The cardiac glycoside ouabain, in concentrations ranging from 2 to 3 $\times$ 10^{-7} M, increases the oxygen uptake of slices of guinea pig heart muscle in the presence of glucose or lactate; at higher concentrations this increase (maximally 50 per cent) is followed by a depression (maximally 80 per cent). Among a variety of other guinea pig tissues studied, brain cortex alone responds in a similar manner, but is only about $\frac{1}{5}$ as sensitive as heart muscle.

2. The oxidation of succinate in cardiac slices is not inhibited at all by ouabain; in brain cortex slices it is inhibited only to a moderate extent. In the presence of pyruvate, the oxygen uptake of cardiac slices, which is high initially, is depressed by ouabain without preceding stimulation. The effect of the drug on the oxygen uptake of brain cortex slices is the same in pyruvate as in glucose or lactate medium.

3. The increase in the respiration of cardiac slices produced by ouabain is dependent upon the presence and concentration of appropriate substrate in the surrounding.

4. The inhibition of the respiration of brain cortex slices produced by ouabain cannot be reversed by washing. On the contrary, placing ouabain-treated, but still actively respiring slices into fresh, ouabain-free medium causes an immediate precipitous fall in activity.

5. Ouabain has no significant effect on the respiratory activity of homogenized heart and brain and of isolated oxidative systems.

6. It is concluded: (a) that ouabain does not directly interfere with the catalytic function of respiratory enzymes; (b) that its influence on the respiration of heart muscle and brain cortex is dependent upon the integrity of cellular structure; (c) that the cell surface is the probable site of action of ouabain on sliced heart muscle and brain cortex, the increase in respiration being due, at least in part, to facilitation of the entry of exogenous substrate in the cell, and the subsequent inhibition to the loss of one or more diffusible respiratory catalysts.

7. These findings and conclusions are discussed in relation to the action of the cardiac glycosides *in vivo*.

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since as time went on refinements in techniques were developed which made possible more careful control of the experimental conditions. In the first test of propylene glycol vapor on rats, a DeVilbiss atomizer # 180 , operated by air under 300 mm. pressure was used to introduce a very fine spray of propylene glycol into the chamber. The droplets produced were sufficiently small so that enough glycol evaporated to maintain a constantly saturated atmosphere. The air pressure supply was regulated automatically by clockwork so that glycol was

TABLE 1

Purity specifications for propylene and triethylene glycols intended for use in aerial disinfection

	PROPYLENE GLYCOL	TRIETHYLENE GLYCOL
Specific gravity	1.0370 to 1.0400 at $\frac{20°}{20}$C.	1.1220 to 1.1270 at $\frac{20°}{20}$C.
Boiling point	Range between 180–210°C., with 90% of the material boiling below 195°C.	275–310°C.
Refractive index	1.4316–1.4335 N_D^{20}	1.4549–1.4565 N_D^{20}
Acidity	Equivalent to not more than 0.093 mg. KOH per gm. sample	Equivalent to not more than 0.19 mg. KOH per gm. sample
Solubility	Completely miscible with water at 20°C., and leaving no insoluble residue	Completely miscible with water at 20°C., and leaving no insoluble matter
Color	Not darker than 15 on the platinum cobalt scale (12)	Not darker than 30 on platinum cobalt scale (12)
Ash	Not more than 0.010% by weight	Not more than 0.010% by weight
Analysis	By periodate oxidation (13) to give at least 95.0% propylene glycol by weight	Hydroxyl value of 22.25–23.00 by means of acetic anhydride-pyridine test
Odor	None	Slight, or none
LD$_{50}$ when administered by stomach tube in 50% aqueous solution	In rats, 22 cc. per kilogram body weight*	In rats, 15–18.0 gm. per kilogram body weight†

* Similar values were found by previous workers (4, 5).

† Similar values were reported by earlier workers (8, 10).

sprayed for 30 minutes out of every hour. This method was eventually abandoned, however, because it was found to be very wasteful of glycol. In its stead, a regulated drip of liquid glycol onto the bare surface of an electric hot plate at about 225°C. was at first substituted. However, this arrangement was also found unsatisfactory as it produced extensive decomposition of the glycol (vide infra). An arrangement which worked fairly well for propylene glycol consisted in a sleeve of glass cloth sewed around the blade of a knife-type electric heater so as to cover it completely. The heater was mounted in a horizontal position above a dish of glycol, into which one end of the glass cloth wicking dipped. Glycol was drawn up into the wick by capillary attraction, and on reaching the

TESTS FOR THE CHRONIC TOXICITY OF PROPYLENE GLYCOL AND TRIETHYLENE GLYCOL ON MONKEYS AND RATS BY VAPOR INHALATION AND ORAL ADMINISTRATION

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The finding that vapors of certain glycols were highly germicidal for air-borne bacteria and viruses (1-3) presented the possibility of employing these compounds for disinfection of atmospheres occupied by human beings. Of the two glycols studied most extensively, namely, propylene and triethylene, the former was known to be essentially non-toxic (4-7) and while acute toxicity tests on triethylene glycol had likewise shown that very large doses are required to kill animals (8-10) the effect of prolonged administration of this glycol had not been determined. Moreover, not withstanding the known inocuousness of propylene glycol when administered by mouth or injected intravenously, it was felt that in the absence of adequate tests we could not assume that the inhalation of this glycol would be without deleterious effect on the lungs. Preliminary intratracheal injections in the rat of as little as 0.25 cc. of undiluted propylene glycol caused marked pulmonary irritation, acute edema and later fibrosis and abscess formation.

The very low vapor pressures of propylene glycol and triethylene glycol at room temperature (table 2) as well as the fact that both glycols are very soluble in the body fluids, made it seem unlikely that in the amounts present as vapor in the air, enough glycol could accumulate to cause irritation of the respiratory tract. Nevertheless it was only as a result of prolonged exposure of animals to atmospheres saturated first with propylene glycol and later with triethylene glycol that this possibility was definitely excluded. A brief report on this work was made several years ago when the study was still under way (11). The present communication embodies the results of the completed investigation.

MATERIALS AND METHODS

Characteristics of Glycols Employed. The glycols employed in these toxicity tests were purified materials which met with the specifications listed in table 1.[1]

Vaporization of Glycols for Inhalation Experiments. Various methods for the vaporization of the glycols were employed, during the course of these experiments

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[1] The glycols used in this study were supplied through the kindness of Mr. D. B. Williams and Mr. E. Fogle of the Carbide and Carbon Chemicals Corporation. This company has placed on the market a purified triethylene glycol labelled "Air Sterilization Grade."

conjunctiva, number of young born and general condition. As shown in table 2 these animals were maintained constantly in an atmosphere supersaturated with propylene glycol vapor.

Growth Rate. The growth curves of the two sets of rats shown in figure 1, were made by averaging the weights of individual animals observed during equivalent periods of time. The weights of the young born in the chambers were added to the growth curve when they reached the value at which their parents began the experiment, e.g. 80 to 90 grams. The weights of only the male rats (20 in the control and 19 in the test group) were plotted since the females varied so much with the birth of young. The animals in the glycol chamber gained weight more rapidly than did the control rats and the weights of the former group at 12

TABLE 2

Atmospheric conditions prevailing in the glycol vapor inhalation experiments

EXPERIMENT	ANIMALS	ROOM TEMP.	RH	CONCENTRATION OF GLYCOL REQUIRED FOR SATURATION	ACTUAL GLYCOL CONCENTRATION
		°F.	%	mg./liter	
Propylene glycol	Rats	75–80	45–65	0.17 –0.35*	Continuous supersaturation
Propylene glycol	Monkeys	78–82	50–60	0.23 –0.35*	About 60% saturation (.10–.22 mg./l.)
Propylene glycol	Monkeys	77–82	50–60	0.23 –0.35*	Continuous supersaturation
Triethylene glycol	Rats	77–82	45–65	0.0025– .005†	Continuous supersaturation
Triethylene glycol	Monkeys	78–82	50–60	0.0031– .0046†	Continuous supersaturation
Triethylene glycol	Monkeys	78–82	50–60	0.0031– .0046†	65–75% saturation (0.002–0.003 mg./l.)

* Calculated on the basis of Raoult's law, using experimental data of Puck and Wise (14) for vapor pressures of pure propylene glycol.

† From experimental data of Wise and Puck (15).

months were about 50 per cent greater than the latter. We have no explanation to offer for this difference since, except for the presence of the glycol vapor, all conditions were approximately the same in each group. There was no essential change in the weights of the rats of either group after twelve months.

The rats in the glycol atmosphere bred just as regularly and produced just as large litters as did the control animals. The young of the two groups were indistinguishable in appearance and weight-gain. No conjunctival irritation was observed. At one period, shortly after the hot plate vaporization was substituted for the atomization method of dispersal, a yellowish discoloration of the coats of the rats in the glycol chamber was observed. This was found to be due to decomposition of the glycol by an excessively high temperature (220°C.) of the surface of the hot plate. When the method of vaporization was changed so that the temperature of the glycol remained below 95°C., the yellow tint of the

zone near the heater, was vaporized. The current through the heater was regulated so that the decomposition temperature of the glycol was not exceeded A fan placed behind the vaporizer distributed the vapor throughout the chamber.

The vaporization of triethylene glycol in these tests was accomplished by a slightly different method, because this compound is more sensitive to thermal decomposition. A satisfactory system for producing atmospheres continuously saturated or supersaturated with triethylene glycol was achieved by placing a shallow dish of the liquid on a hot-plate which was regulated to keep the temperature of the glycol at 100°C. A constant level device attached to a larger reservoir prevented loss of volume of the liquid in the dish, with consequent rise in temperature. A small electric fan behind the vaporizer, dispersed the vapor throughout the test chamber. In the final test where the concentration of triethylene glycol vapor was kept constant by means of a glycostat (21) this same principle of vaporization was employed, except that the vaporizer was enclosed in a metal cylinder at the top of which a magnetically-controlled butterfly valve was arranged. The glycostat, responding to the actual concentration of glycol vapor in the air, automatically adjusted the butterfly valve so as continuously to maintain the desired vapor concentration. This last method, which operated continuously for ten months provided the most precise and convenient means for producing a controlled vapor concentration, over long periods of time.

A summary of the atmospheric conditions which prevailed in the tests which will be described is presented in table 2. In considering the concentrations of each glycol vapor attained in the air, cognizance must be taken of the variation in the saturation point with relative humidity (15). In all the experiments where the glycol level is listed as "continuous supersaturation," so much excess vapor was introduced that the room was constantly filled with a dense fog of condensed glycol droplets. This condition would represent an amount of glycol greatly in excess of that regarded as desireable for aerial disinfection under conditions of human habitation (16).

PROPYLENE GLYCOL

Inhalation of Vapor by Rats. The first tests on the effect of inhaling propylene glycol vapor were made on white rats. An initial colony of 30 animals weighing 80 to 90 grams at the beginning of the test were divided into two groups—20 test and 10 controls. The animals were placed in identical chambers 5' long, 3' high and 2'6" deep with 5–6 individuals in a cage. The number of rats in each group was increased by birth of young. Breeding was controlled to produce about equal populations in the two groups. The temperature in the chambers was maintained between 75 and 80°F. by means of a fan blowing on a small radiator through which flowed a controlled stream of tap water. By using large trays of $CaCl_2$ it was found possible to prevent the relative humidity exceeding a range of 45 to 65 per cent.

The rats were fed on a dry meal diet consisting of corn meal, linseed meal, casein, powdered alfalfa, powdered brewers yeast, $CaCO_3$ and NaCl. Observations during life were made on gain in weigh, color of coat, possible effects on

each lobe of the lungs was taken as well as sections of liver, kidney and spleen. Maximow's hemotoxylin-eosin-azure stain was employed.

Lungs. The gross appearance of the lungs in both the glycol exposed and control rats was normal except in certain instances in which consolidation of a part or a whole lobe was present. On microscopic examination of such regions, evidence of a localized infectious process was found. This varied from a very small area of intra-alveolar accumulation of polymorphonuclear leucocytes to the involvement of a whole lobe. In some cases the lesion was in the process of resolution as shown by the macrophagic nature of the exudate. Such lesions were with a single exception found only in rats which had been kept in the chambers for 8 months or longer[2] and occurred in 25 per cent of the control animals (table 4). In contrast only two lesions of a similar nature were found in the 26 rats kept in a propylene glycol atmosphere for 8 to 18 months.

The most common change in the lungs was a pervascular and peribronchial accumulation of round cells which first began to appear after the end of $4\frac{1}{2}$ to 5

TABLE 3

Exposure of rats to propylene glycol vapor

	NUMBER OF MONTHS OF EXPOSURE TO TEST CONDITIONS														TOTAL NUMBER OF RATS
	3	4	5	6	7	8	9	10	11	12	13	15	17	18	
Number of rats killed in each chamber at successive intervals															
Glycol	1	4	5	2	1	3	3	2	0	3	8	3	0	4	39
Control	2	3	2	4	2	0	5	2	1	3	0	4	3	7	38

months residence in the chambers. As time went on the size of these "collars" of cells increased and in certain animals became most pronounced. This change occurred with equal frequency in both control and test animals. Figure 2 shows perivascular collars of moderate degree in a 5 months-old control rat. Examination of rats from other sources has revealed the same appearance of the lungs which is most pronounced in old animals. Except for these cellular changes which presumably indicate the occurrence of chronic irritation of some kind and the occasional focal pneumonitis, the lungs of both groups of rats presented a normal appearance.

Other Organs. Examination both gross and histological of the kidneys, liver and spleen revealed no pathological changes. No concretions were found in the bladder or calices of the kidneys except in one instance, that of a control rat which exhibited an oblong stone, 1 cm. long and 0.6 cm. in diameter in the bladder.

Inhalation of Propylene Glycol Vapor by Monkeys. It seemed advisable also to test the effect of inhalation of glycol vapors on monkeys since their upright

[2] In only one out of 51 rats, 3 to 7 months old, was a pulmonary lesion found.

coat disappeared. All the rats, both glycol-exposed and normal, appeared to be in good condition at the time they were sacrificed. No deaths occurred.

Examination at Autopsy. After intervals of time from 3 to 18 months as shown in table 3, the rats were killed with an intra-peritoneal injection of pentobarbital sodium. Urine aspirated from the bladder showed no abnormal findings

FIG. 1. COMPARATIVE GROWTH RATES OF RATS KEPT CONTINUOUSLY IN AN ATMOSPHERE SATURATED WITH PROPYLENE GLYCOL VAPOR AND A SIMILAR GROUP OF CONTROL ANIMALS

Male rats only included in the graph; nineteen in the test and twenty in the control groups. Rats about seven weeks old when experiment begun.

in either test or control animals. The lungs were fixed with Zenker-formol by the method previously described (17) which consists in clamping off the aorta, next compression of the ventricles in order to fill the pulmonary vessels, followed by application of a tight suture around the base of the heart. The fixing fluid is then allowed to run into the trachea under slight pressure. One section through

position would provide the optimum opportunity for the condensed glycol to remain in the lungs. The lack of deleterious effect found in rats could conceivably be attributed to the more effective elimination of this material from their lungs due to the horizontal position of the air passages (18).

Forty-five Macacus Rhesus monkeys were secured from the tuberculosis-free colony of Columbia University at San Juan, Puerto Rico.[3] Twenty-nine were exposed to propylene glycol vapor and sixteen employed as controls.

Three insulated air-conditioned chambers 9 x 6.5 x 8 feet high were constructed for the experiment in which temperatures of 78 to 82°F. and relative humidities of 50 to 60 per cent were constantly maintained. The air flow through each chamber amounted to 13 complete changes of air per hour. The cages made of heavy 2" mesh steel wire were 36 x 28 x 39 inches high. Since the monkeys were small, three to four were able to live comfortably in a single cage. The two glycol

TABLE 5

Exposure of monkeys to propylene glycol vapor

| | NUMBER OF MONTHS OF EXPOSURE TO TEST CONDITIONS | | | | | | | | | | | TOTAL NUMBER OF MONKEYS |
	1	2	3	4	5	6	7	9	11	12	13	
Number of monkeys autopsied in each chamber*												
Glycol..... .	1			6	3	7	2	1	2	1	6	29
Control....	2		1		8	1	4					16

* Monkeys died or were sacrificed at termination of exposure times indicated in table.

chambers contained 14 and 15 monkeys respectively and the control 16. The diet consisted of oranges, apples, potatoes, bananas, carrots and bread to which was added egg-nogs containing vitamin B complex and cod liver oil. No ultraviolet radiation was employed.

Propylene glycol vapor was dispersed in two of the chambers as shown in table 2. Supersaturation of the air with glycol vapor was present constantly in one chamber while in the other a concentration of about 60 per cent saturation was maintained.

The lengths of time the monkeys were kept in the test and control chambers is shown in table 5. An x-ray of the lungs was made shortly after the monkeys arrived. They also were tuberculin-tested by means of the intracutaneous injection of O.T. 1–1000 into the soft tissue lateral to the eye. All x-rays and tuberculin tests were negative. These two procedures were repeated at the end of the experiment. The animals were weighed at monthly intervals and observations made on the texture and color of hair and skin, condition of eyes, appetite, activity and any abnormal signs or symptoms. Complete blood counts were made at the beginning of the experiment and again just before they were sacri-

TABLE 4

Number of pulmonary lesions occurring in rats both exposed and unexposed to atmospheres containing propylene or triethylene glycol vapors

LENGTH OF TIME OBSERVED	RATS USED FOR	TOTAL NUMBER OF RATS	NUMBER SHOWING PULMONARY LESIONS	PER CENT TOTAL SHOWING PULMONARY LESIONS
months				
	Inhalation of propylene glycol	26	2	8
	Controls on inhalation of propylene glycol	25	6	24
8–18				
	Inhalation of triethylene glycol*	34	8	24
	Controls on inhalation or ingestion of triethylene glycol	44	12	27
3–7	Inhalation of propylene glycol	13	0	0
	Inhalation of triethylene glycol	25	1	4
	Controls on inhalation or ingestion of glycols	13	0	0

* This group included animals considerably older than those in the propylene glycol group and it is probable that lung lesions had occurred in some before the experiment began.
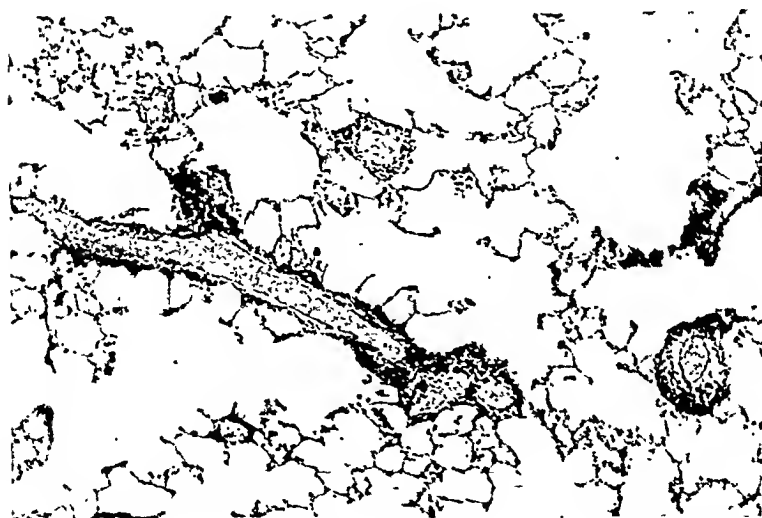
FIG. 2. PHOTOGRAPH OF THE LUNG OF A CONTROL RAT FIVE MONTHS OLD

Each blood vessel is surrounded by a collar of cells (mononuclear) which appears to be almost continuous along the vessel wall. The remainder of the lung architecture is normal. Magnification × 125.

Tests for possible impairment of kidney function were made by determining their ability to concentrate the urine during a period of 24 hours in which both
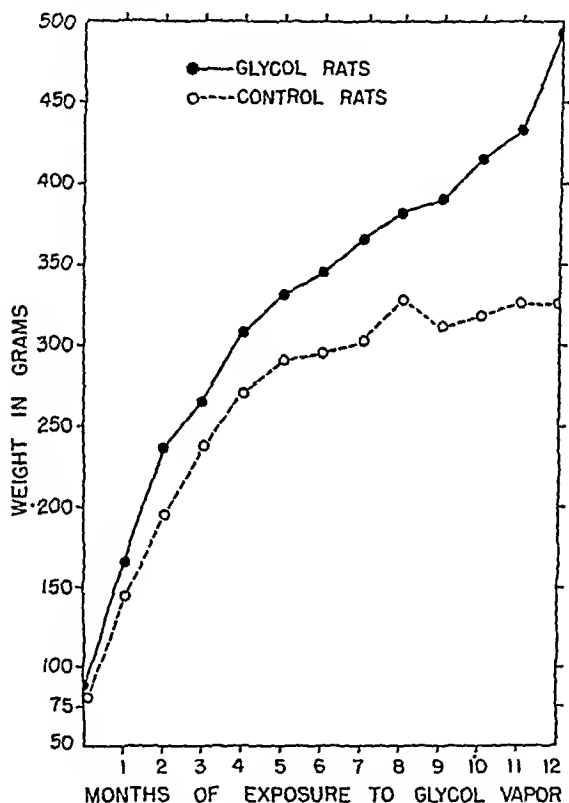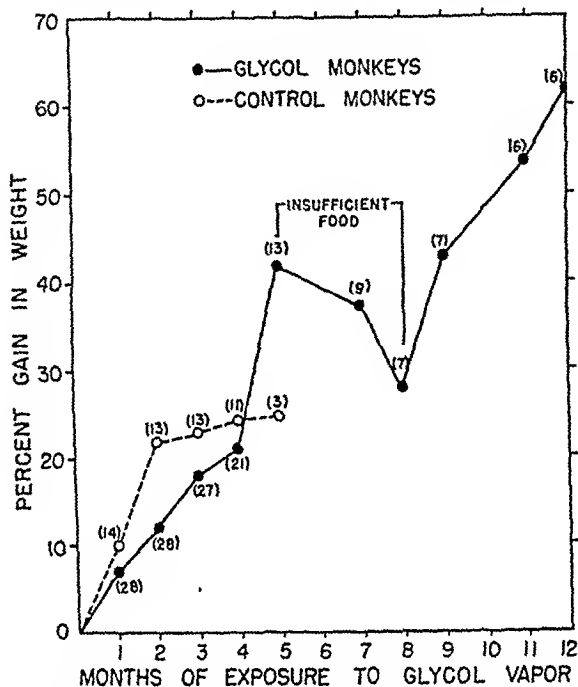

FIG. 3. COMPARATIVE GROWTH RATES OF MONKEYS KEPT CONTINUOUSLY IN AN ATMOSPHERE SATURATED WITH PROPYLENE GLYCOL VAPOR AND A GROUP OF CONTROLS

Average initial weights of monkeys in glycol group 2959 grams and in control group 2459 grams. The figures in parenthesis indicate number of animals in each weighing.

TABLE 7

Blood counts and hemoglobin determinations of monkeys exposed to propylene glycol vapor

| | TIME OF DETERMINATIONS | RED BLOOD CELLS | WHITE BLOOD CELLS | HEMOGLOBIN | DIFFERENTIAL | | | | |
					Polynuclears	Lymphocytes	Monocytes	Eosinophiles	Basophiles
Glycol group	Initial	2.58	15.9	11.9	56	27	11	6	1
	Final	5.18	22.3	13.4	71	18	7	9	1
Control group	Initial	2.61	18.8	11.6	44	29	17	7	1
	Final	4.77	26.1	11.9	67	17	10	1	1

water and food were withheld. Both groups of monkeys concentrated their urine equally well under these conditions. Microscopic examination of the

ficed. Tests for the ability of the kidneys to concentrate urine were conducted at the end of the period of observation.

Within the first seven months, three of the sixteen monkeys in the control chamber and seven of the 29 animals in the glycol chamber died. Eight other monkeys were sacrificed at the time they were very ill in order to obtain antimortem tissues. The distribution of these deaths and infections is shown in table 6.

The weights of the two groups of animals are shown in figure 3. Final weights of sick monkeys just before being sacrificed are not included. Unfortunately, the weight gains in the two groups of monkeys could not be compared for longer than five months due to an insufficient number of remaining controls. Except for the period from five to eight months when the monkeys in the glycol cham-

TABLE 6

Occurrence of disease and death in control and propylene glycol-exposed monkeys

	NUMBER SUCCUMBING TO INFECTION FROM					TOTAL NUMBER DEATHS	NUMBER REMAINING WELL UNTIL SACRIFICED
	Nematode infection	Bacterial infection	Dysentery	Malnutrition	Cause unknown and injury		
Glycol group (29 animals)							
Died . . .	2	2 oral	1	1	1 cause unknown	7	
Sacrificed when very ill	3	2 oral	1			6	16
Control group (16 animals)							
Died . . .	2				1 cause unknown	3	
Sacrificed when very ill	5	1 oral			1 injury	7	6

bers lost weight (found to be due to insufficient food)[4] these animals gained weight at a normal rate until they were sacrificed at twelve months.

The results of blood counts and hemoglobin determinations are shown in table 7. Upon arrival in the laboratory the monkeys were all found to be suffering from a considerable degree of anemia. That this was probably not due entirely to the round worm infection (described below) is shown by the fact that on a liberal diet containing ample vitamins the blood picture had improved markedly by the time these animals were sacrificed. Restoration of the higher level occurred within three to four months and thereafter the blood picture remained essentially unchanged. The blood of the monkeys maintained in the glycol atmosphere showed a slightly greater increase in red blood cells and a distinctly higher hemoglobin content than did the control animals.[5]

[4] At about the 6th month the quantity of food was reduced to the amount which the animals would entirely consume. This was found to be mistaken economy since restoration of an excess supply of food following the 8th month weighing resulted in a rapid and continuous gain in weight.

[5] We wish to record our indebtedness to Dr. Leon O. Jacobson, Department of Medicine, University of Chicago, and his laboratory staff for carrying out these red counts and hemoglobin determinations.
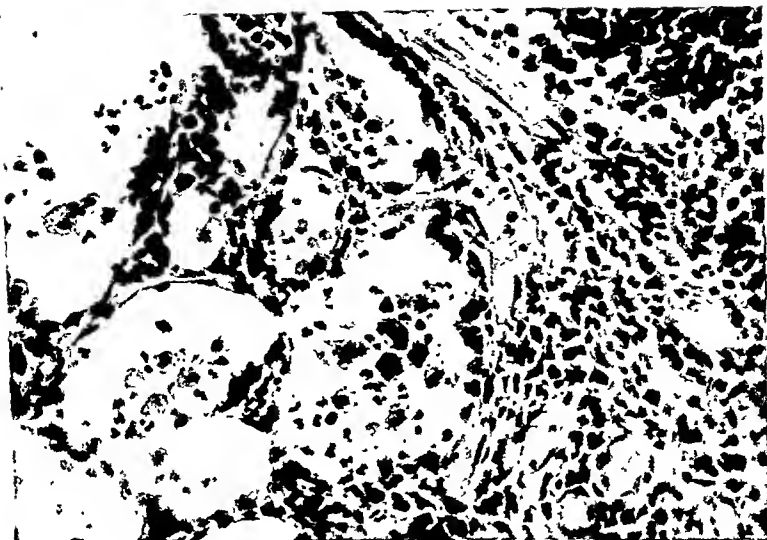
FIG. 4. HIGH POWER VIEW OF A PORTION OF THE WALL OF THE LUNG
MITE CYST WHICH SHOWS A DENSE ACCUMULATION OF
MONONUCLEAR AND POLYMORPHONUCLEAR LEUCOCYTES

In the adjacent alveoli macrophages and polymorphonuclear leucocytes are present. In places the septal cells are increased in number Magnification ×480.

FIG. 5 PHOTOGRAPH OF A LUNG MITE (PNEUMONYSSUS GRIFFITHI)
CYST SHOWING A LONGITUDINAL SECTION OF THE PARASITE
LYING IN THE LOWER PORTION OF THE CAVITY

The head is toward the left hand side. Beyond the head is a collection of exudate cells which partly surround a fragment of another mite. Magnification ×82.

urine showed occasional hyaline casts, a few white blood cells and sometimes a slight trace of albumin. No differences were found between the controls and test monkeys.

In addition to those monkeys sacrificed at a time when they were very ill, certain well ones in both control and test groups were killed from time to time. All these are included in the figures of table 5. The animals which remained well showed no change except that certain ones in the glycol chambers developed brownish discoloration of the face after a few months. This cleared up following a week or two's sojourn outside the chamber and was considered to be due to the drying effect of the glycol on the skin. Their coats remained glossy and they were very active.

Examination at Autopsy. Two pathological conditions were found in practically all the monkeys both test and control: (1) infection with Aesophagostomum apiostomum, a parasitic nematode closely related to the hookworm, and (2) infestation of the lungs with the lung mite Pneumonyssus griffithi. On opening the abdomen, small chocolate colored cysts, 3–5 mm. in diameter, enclosing the larval worms, were scattered throughout the omentum and along the wall of the bowel. These varied from a few to many scores. Adhesions were frequently present, and were most extensive in animals dying of the disease where they occasionally caused obstruction of the common duct, or interference with the portal circulation as evidenced by ascites, or partial intestinal obstruction. Adult worms were found in the intestinal contents. The monkeys with severe infection showed malnutrition and sometimes marked anemia.

The lungs of almost all the animals showed numerous small whitish nodules, 2–5 mm. in diameter, both on the surface and throughout the lung. On microscopic examination they were found to consist of a central cavity surrounded by a dense cellular wall which was composed mostly of macrophages though in places numerous polymorphonuclear leucocytes were present (fig. 4). In some of the cavities the mite was present as shown in figure 5. No evidence of tuberculosis was found nor were any other pathological changes detected in the lungs of either the glycol-exposed or the normal monkeys. The lungs were fixed by the method employed in the preparation of the rats' lungs. Two sections were made through each of the principal five lobes of the lungs and one through the small subcardiac lobe—eleven sections in all.

With the above exceptions the gross appearance of the organs of the thoracic and abdominal cavities was normal. Microscopic examination of the liver, kidneys, spleen, mesenteric glands, adrenals and in certain cases stomach, in-intestines and testes showed no differences between test and control monkeys. No stones were found in the kidneys or bladder. In the animals which died from disease or were killed when very ill (table 6) certain other changes characteristic of the disease process were found, e.g hemorrhagic bowel wall in those dying of dysentery.

The tests on exposing monkeys and rats continuously to atmospheres saturated with propylene glycol vapor for periods of 12 to 18 months may be summarized by the statement that no deleterious effects either functional or organic could be

difference in the growth rates of the two groups. After this age, however, the rats in the glycol atmosphere continued to grow while the weights of those in the control group remained almost stationary. The animals in the two groups appeared in excellent condition. The young born in the glycol atmosphere seemed to be normal in every way, and gained weight just as rapidly as did the controls of the same age.
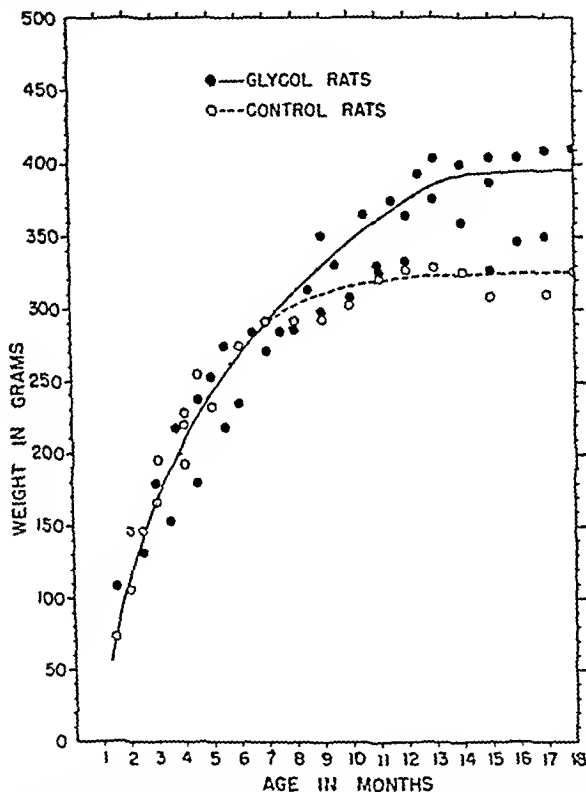
FIG. 6. COMPARATIVE GROWTH RATES OF RATS KEPT CONTINUOUSLY IN AN ATMOSPHERE SATURATED WITH TRIETHYLENE GLYCOL VAPOR AND A SIMILAR GROUP OF CONTROL RATS
Male rats only included on the graph

Blood Examination. Complete blood counts and hemoglobin determinations were made on each rat at the end of the time of residence in the vapor and control chambers and are exhibited in table 9. After 8–13 months continuous exposure to a fog of triethylene glycol vapor, rats showed no differences in their blood picture from that of the control animals.

Ingestion of Triethylene Glycol by Mouth. Four groups of eight rats each weighing between 200 and 300 grams were employed as shown in table 8. The

attributed to this treatment. Actually both the rats and monkeys kept in the glycol atmosphere appeared to do somewhat better than the control animals in respect to gain in weight, incidence of pulmonary infection and increase in red blood cells and hemoglobin.

TRIETHYLENE GLYCOL

Inhalation of Vapor by Rats. Thirty-six rats ranging from 100 to 350 grams in weight, two-thirds of which were males, were placed in a chamber containing glycol vapor and 6 rats in another which served as the control. The populations were increased during the experiment by the birth of young to 60 in the vapor and 14 in the control groups (table 8). As shown in table 2 the rats in the glycol group were maintained continuously in an atmosphere supersaturated with triethylene glycol vapor.

TABLE 8
Rats exposed to triethylene glycol vapor and given the glycol orally

| | NUMBER OF MONTHS EXPOSURE TO TEST CONDITIONS | | | | | | | | | | | | TOTAL NUMBER OF ANIMALS |
	$\frac{1}{2}$	3	4	5	6	7	8	9	10	11	12	13	
Number of rats killed in each group at successive intervals Vapor .	0	10	10	0	5	0	26	1	0	0	5	3	60
Oral 35X* .	0	0	0	2	0	0	4	0	0	0	0	2	8
80X . .	1	0	1	2	0	0	3	0	0	0	0	2	9 45
700X ..	0	4	0	2	0	1	3	0	3	0	3	12	28
Control .	0	0	0	2	0	0	3	0	0	0	5	4	14

* 35X, 80X and 700X = the calculated dosage in terms of the maximum amount of triethylene glycol vapor a rat could inhale in 24 hours in an atmosphere saturated with this substance.

Growth Rates. Since the rats employed in this test varied in age from six weeks to six months at the beginning of the experiment it was not possible to plot the growth curve in the same manner as that exhibited for the propylene glycol rats which were all initially of the same age. Each dot on the graph (fig. 6) represents the average weight of a group of rats consisting of from four to 14 animals. Certain of the 2-3 months-old rats shown in the graph were carried through to the termination of the test (13 months). The older rats were added to the graph at their respective ages when started in the test. There were 36 animals exposed to glycol and 46 controls.[6] Only male rats are included in the graph. It will be noted that for the first seven months there was essentially no

[6] For purposes of the weight chart the male control rats of the propylene glycol experiment plus half a dozen normal rats kept under usual room conditions were combined with the triethylene glycol control animals. All the rats were fed the same diet.

hours) were, in the latter half of the experiment allowed to breed. They had frequent large litters which increased this population from 8 to 28.

All these animals remained in good condition. The weight gains of the four groups of animals were approximately the same (table 10). Examination of the blood at the end of the test period showed no essential differences between the glycol-fed and the control rats (table 9). Microscopic examination of the urine during life revealed no abnormalities.

Examination at Autopsy. The post mortem findings in rats exposed to tri-ethylene glycol vapor and drinking the glycol were essentially the same as those of the rats exposed to propylene glycol; i.e. there were no pathological changes except focal areas of pneumonitis. As shown in table 4 these lesions were present in approximately the same percentage of test animals and controls.

Inhalation of Triethylene Glycol Vapor by Monkeys. First Test—Super-saturated Glycol Atmospheres. Twenty-five monkeys from the Puerto Rico colony were divided into two groups. Seventeen were maintained in a fog of triethylene glycol, eight were kept in one of the other chambers as controls. The atmospheric conditions and concentration of glycol vapor are given in table 2. The glycol supersaturation was sufficiently high to produce a dense fog which caused continuous condensation on all surfaces inside the chamber. The other conditions under which they were maintained were the same as those described for the monkeys exposed to propylene glycol vapor. X-rays of the lungs and tuberculin tests carried out at the inception of the experiment were all negative. The number of months the animals were kept in the test is shown in table 11.

As table 12 shows there was a considerable morbidity and mortality from the same causes which affected the former group of monkeys. The animals in the control group gained weight somewhat more rapidly than did those in the glycol atmosphere (fig. 7) and at the end of eight months had shown a weight gain equivalent to 66 per cent of their initial weight while those in the glycol had gained only 44 per cent. Even by the end of 13 months the glycol monkeys had gained not quite 60 per cent of their initial weight.

Another difference between the glycol-exposed and the control group was the appearance of a browning of the skin of the face which occurred in the former animals after 3 to 4 months in the glycol atmosphere. This was of the same nature as that observed in the case of the monkeys exposed to propylene glycol vapor, and disappeared within a few days after removal to glycol-free air. Some of the animals exposed to triethylene glycol vapor also showed crusting of the ears with thickening of the lateral edge of the ear and actual loss of tissue in certain instances. Microscopic sections of these tissues revealed the presence of numerous cysts containing an unidentified ectoparasite. Such lesions were only very occasionally seen in the control monkeys and were not nearly as pronounced. It is possible that the effect on the skin caused by the supersaturated glycol atmosphere rendered the integument of the ears more susceptible to this form of parasitic invasion. Furthermore, the fact that these monkeys were observed to scratch and pull at their ears constantly, suggested the possibilty of the implantation of the parasite by this means.

glycol was added to the drinking water in amounts calculated in terms of multiples of the maximum quantity of glycol a rat could inhale in vapor form if kept in a saturated atmosphere for 24 hours. This quantity is approximately 0.004 cc. of glycol per kilogram of rat per day.[7] Preliminary tests showed that individual rats of the same weight drank about the same quantity of water each day. The quantity of glycol solution consumed in each cage of two rats was measured at

TABLE 9

Blood counts of rats after exposure to triethylene glycol vapor and oral ingestion of the glycol

	LENGTH OF EXPOSURE	RED BLOOD CELLS	WHITE BLOOD CELLS	DIFFERENTIAL COUNT				
				Polynu-clears	Lym-pho-cytes	Mono-cytes	Eosino-philes	Baso-philes
	mo.	*millions*	*thousands*					
Glycol vapor (57 rats).....	6–13	7.61	15.2	32	56	5	6	>0.5
Controls—vapor (17 rats).	6–13	7.51	12.1	28	66	3	2.5	0.5
Oral ingestion dose								
(7 rats) ×35...........	5–13	5.87	14.5	35	56	6.4	2.6	0
(8 rats) ×80	5–13	6.11	11.5	28	62	7.2	2	0.5
(24 rats) ×700... ..	5–13	6.14	12.0	35	60	2	3	0
Controls—oral (9 rats)...	12–13	5.62	12.1	32.5	56.5	5.5	5.5	0

TABLE 10

Amounts of triethylene glycol ingested per day per rat in the different groups exhibited in table 8

	DOSAGE GROUPS OF RATS			CONTROLS
	35×	80×	700×	
Total fluid per month.......	708 cc.	784 cc.	712 cc.	836 cc.
Total glycol per month.....	0.85 cc.	1.96 cc.	16 cc.	0
Glycol per day......	0.028 cc.	0.065 cc.	0.533 cc.	0
Glycol per day per kilograms of rat........	0.14 cc.	0.32 cc.	2.66 cc.	0
Average weight, in grams, at				
Beginning of experiment..	264	292	246	260
End of experiment.......	334	363	328	305

the end of each week. This data is exhibited in table 10. The dosage indicated for each group represents the amount of glycol actually consumed which is somewhat less than that expected from the initial calculations. Since it seemed desirable to secure the most information about the largest dose of glycol, the rats on 700× (i.e. ingesting approximately 700 times the quantity inhaled in 26

[7] These calculations were only approximate as they were based on the actual measurement of the tidal air in 18 gram mice which was 0.25 cc. (19). The rats tidal air was estimated to be ten times this figure and the 24 hour air volume was calculated on the basis of 75 respirations per minute. The average glycol concentration corresponds to 0.004 mg./l.

egg nog which they always drank readily. The daily and total quantities ingested are shown in table 14. The daily oral dosages given, 0.25 cc. and 0.5 cc., were calculated as representing approximately 50 and 100 times that amount which a monkey could inhale during 24 hours sojourn in an atmosphere saturated with the glycol vapor.[8]
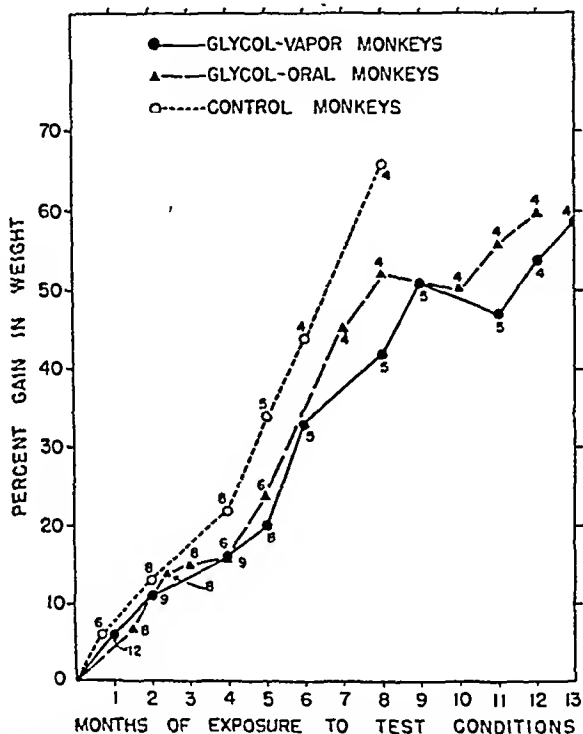
FIG. 7. COMPARATIVE GROWTH RATES OF MONKEYS KEPT CONTINUOUSLY IN AN ATMOSPHERE SATURATED WITH TRIETHYLENE GLYCOL VAPOR (TEST NO. 1), MONKEYS INGESTING THE GLYCOL AND A CONTROL GROUP

Average weights of the different groups at the beginning of the experiment were: glycol vapor 2276 grams, glycol ingestion 2036; control 1973. Figures in parenthesis indicate number of animals in each weighing.

The weight gain of these animals shown in figure 7 was approximately the same as that of the monkeys in the glycol atmosphere and a little less than that of the control group. These animals all remained healthy and one gave birth to a live

[8] The tidal air of the monkey was calculated from that of the human being in relation to weight. In this case approximately ½₀ of the tidal air of human beings or 25 cc. per respiration. Calculations were based on 30 respirations per minute and a concentration of glycol of 0.001 mg./l. This gave a 24 hour total of about 0.0045 mg. of triethylene glycol.

In other respects, however, the findings on monkeys in the glycol atmosphere were essentially the same as those of the control animals. The red blood counts and hemoglobin determinations showed a decline in both groups of animals to about the same degree (table 13, Test no. 1). This was probably caused by the presence of nematode infection. No significant differences in the total number of white blood cells or differential were found. Repeated examination of the urine showed no albumin, sugar or microscopic elements in most of the animals and a slight trace of albumin, an occasional granular and hyaline cast or a few white

TABLE 11

Exposure of monkeys to triethylene glycol vapor test number 1

| | NUMBER OF MONTHS OF EXPOSURE TO TEST CONDITIONS | | | | | | | | | | TOTAL NUMBER OF MONKEYS |
	1	2	3	4	5	6	7	8	9	12	
Number of monkeys autopsied in each chamber*											
Glycol.................	4		2	3		3				5	17
Control.....					2	1	1	4			8

* Monkeys died or were sacrificed at termination of exposure times indicated in table

TABLE 12

Occurrence of disease and death in control monkeys and those exposed to triethylene glycol vapor test no. 1

| | NUMBER SUCCUMBING TO INFECTION FROM | | | | TOTAL NUMBER DEATHS | NUMBER REMAINING WELL TILL SACRIFICED |
	Nematode infection	Bacterial infection	Dysentery	Injury or cause unknown		
Glycol group (17 animals)						
Died......................		2		2 injury	4	
Sacrificed when very ill.....	2		1		3	10
Control group (8 animals)						
Died......................	1				1	
Sacrificed when very ill.....	4				4	3

blood cells in some of them. Both groups of animals showed equal ability to concentrate urine from a specific gravity of 1.010 to 1.030 or 1.040. These tests were conducted soon after the beginning of the experiment and again just before the animal was sacrificed.

Except for these animals which died or were killed because they were very ill (table 12) the monkeys in both groups were very active, ate well and had smooth glossy coats.

Oral Ingestion of Triethylene Glycol by Monkeys. Eight monkeys from the same shipment as those used for exposure to triethylene glycol vapor in Test no. 1 plus two others (older ones) were given the glycol orally by adding it to

the test animals. Gomeri's (20) phosphotase stain of the kidneys showed a heavy precipitation along the secretory portion of the tubules indicating good functional activity. The bone marrow was very cellular, appeared normal and exhibited both the myelogenous and erythroid series. No evidence of tuberculosis or any sign of chronic irritation (except for that due to the lung mites) was found in the lungs. The other organs, spleen, liver, stomach and intestines were likewise normal except for the presence of nematode cysts in the intestinal walls and mesentery.

In animals dying during the course of the experiment for causes indicated in table 12, pathology peculiar to the particular infection was found, which in no way could be ascribed to any toxic effect of glycol since such lesions were present in the controls as well as in the test animals.

Inhalation of Triethylene Glycol Vapor by Monkeys. Second Test—Glycol Maintained Below Saturation with Glycostat. Since the monkeys kept in an atmosphere supersaturated with triethylene glycol vapor did not gain weight quite as well as did the control animals and also showed certain effects on the skin, such as drying and discoloration, it was felt that a second test should be conducted under conditions approximating more nearly those which would be desirable for the use of glycol vapor in human habitations. The lessened gain in weight might have been due to a greater but undetectable degree of nematode infection in the test group, but the fact that the monkeys ingesting glycol also failed to grow as rapidly suggested that glycol was probably the important factor. Since no pathology was detectable in animals drinking 100 times as much glycol as they could possibly ingest from exposure to vapor alone, it seemed likely that the lesser weight gain in these animals might be attributable to a depressant action of the glycol on the appetite. In the case of those living in the fog, a similar effect might be ascribed to an unfavorable environment.

At the time the first test was carried out we had available no means of controlling the concentration of glycol vapor in the atmosphere. The only way of insuring the presence of a bactericidal concentration was to maintain a slight fog. The subsequent development of the glycostat (21) made it possible to carry out another experiment in which monkeys could be kept in an atmosphere free from fog but at the same time containing bactericidal concentrations of triethylene glycol.

The monkeys, obtained from a source other than that of the two previous tests, were free from peritoneal nematodes, lung mites and tuberculosis (with one exception). The group was composed of sixteen animals about two years old, half of which was used for the test and half for controls. The triethylene glycol was dispersed as in the previous experiment except that the output of the vaporizer was controlled by a glycostat which was set to maintain the concentration of the glycol vapor in the air from 65 to 75 per cent saturation (see table 2). No fog was visible at any time. All other conditions were kept the same as described in the previous experiments. These monkeys were kept under test conditions for ten months.

Within a month it was observed that one monkey in the glycol group failed to

normal-appearing infant at the end of four months during which time she had ingested 64.5 cc. of triethylene glycol. The baby monkey lived for two months and seemed to do well but was found dead one morning. Autopsy revealed no cause for death.

Examinations of the blood and urine of these monkeys yielded practically identical results with the controls except that the monkeys taking the glycol by mouth showed less anemia at the end of the test period (table 13).

TABLE 13

Blood counts and hemoglobin determinations at beginning and end of exposure of monkeys to triethylene glycol vapor and oral ingestion

MONKEYS EXPOSED	RED BLOOD CELLS (MILLIONS)		WHITE BLOOD CELLS (THOUSANDS)		HEMO-GLOBIN (GM.)		WHITE BLOOD COUNTS									
							Poly-nuclears		Lympho-cytes		Monocytes		Eosino-philes		Basophiles	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Test no. 1																
Glycol vapor (fog) ..	5.20	4.30	19.70	18.32	11.9	11.5	45.23	73.59	40.71	20.0	7.28	5.71	6.0	1.68	1.33	1.0
Control . . .	6.00	4.30	22.95	19.53	12.3	11.1	54.85	71.85	35.28	23.42	3.16	2.80	5.71	1.50	2.0	1.0
Oral ingestion .	5.27	4.99	20.36	20.37	11.3	12.8	65.23	79.25	23.50	13.25	5.75	5.62	3.0	2.0	1.25	1.00
Test no. 2																
Glycol vapor (no fog) .	5.45	6.00	23.26	—	14.3	92%*	44.0	—	53.50	—	—	—	2.33	—	—	—
Control	5.39	5.90	27.52	—	14	87%*	53.71	—	45.0	—	—	—	2.0	—	—	—

* These determinations made on Dare Hemoglobinometer.

TABLE 14

Monkeys taking triethylene glycol orally

NUMBER OF ANIMALS	DOSE OF TEG* DAILY	DURATION OF INGESTION	TOTAL AMOUNT OF TEG INGESTED
	cc.	*mo.*	*cc.*
2	0.5	3	45
2	0.5	5½	83
4	2 = 0.25	12	2 = 91
	2 = 0.5		2 = 183
2	0.5	14½	220

* Triethylene glycol.

Autopsy. Since the findings at autopsy of all three groups of monkeys, i.e. those exposed to the glycol vapor, those drinking glycol and the controls, were essentially the same, they will be described together. With the exception of two animals in the oral-ingestion group which were obtained from another source, the monkeys all showed lung mites and varying degrees of round worm infestation. The two animals above mentioned showed neither of these parasites.

Examination, both gross and microscopic, of the different organs—lungs, liver, kidneys, spleen, bone marrow, revealed no differences between the control and

glycol atmosphere showed any discoloration of the skin of the face or any change in the color or texture of the ears. They were all lively, exhibited glossy coats and gained weight about equally well with the exception of one of the test animals which showed a relatively small increase in weight.

Autopsy. Since this second experiment with triethylene glycol was conducted primarily for observations on weight-gain and skin effects, it was not considered necessary to do post-mortem examinations in all the monkeys in view of the completely negative findings in respect to any deleterious effect of the glycol in animals of the first test group. Three animals were sacrificed, two from the glycol group and one control. The internal organs were entirely normal with the exception of one of the test animals which exhibited a localized area of chronic gastritis at the pyloric end of the stomach. This was the single monkey which had shown an unsatisfactory weight gain from the beginning of the experiment.

In summarizing the above described tests on the toxicity of triethylene glycol for monkeys and rats it may be stated that among the large number of animals studied no pathological changes, ascribable to effect of the glycol were detected. The only disturbances which could be related to the presence of triethylene glycol vapor in the air were drying of the skin and slight interference with nutrition. That these two effects were due to an excess of glycol vapor (fog) was indicated by their absence in the second experiment in which the concentration of glycol was kept below saturation by means of the glycostat.

DISCUSSION

Two studies on the prolonged administration of propylene glycol have been reported, one by Seidenfelt and Hanzlik (7) the other by Morris, Nelson and Calvery (22). The animals tested—rats—either drank water containing 10 per cent propylene glycol or ingested the glycol incorporated in the food in quantities of 2.5 to 4.9 per cent of the total food weight. In the latter experiment the animals showed no ill effects after two years on such a regime. The quantities of glycol administered to these animals daily was several hundred times the amounts estimated to have been inhaled by the rats in our study. The only new information contributed by the present investigation of the toxicity of propylene glycol is the demonstration that the continuous residence of monkeys and rats for a year or more in an atmosphere supersaturated with the vapor of this glycol was without deleterious effect on the lungs or functional activity of the body as a whole. In fact the animals in the glycol atmosphere seemed to thrive somewhat better than the control groups as judged by weight gain, and increase in red blood cells and hemoglobin content.

On the other hand there are no data available on long term toxicity studies with triethylene glycol. Tests carried out previously have all been of an acute nature except those of Lauter and Vler (9). These authors found that young rats drinking water containing 3 per cent triethylene glycol showed no ill effect after 30 days. Adult rats given water containing 5 per cent of the glycol died—although the young rats survived. While the maximum concentration of glycol in

gain weight. During the succeeding month it became ill and died. Autopsy revealed widespread pulmonary tuberculosis. In spite of the fact that the tuberculin tests on the monkeys shortly after reaching the laboratory were all negative, it seems probable that the infection was acquired before arrival in Chicago since x-rays of the lungs of the caretaker and all other persons who had contact with the monkeys were normal. The cage mate of this monkey remained well, gained weight and showed no evidence of pulmonary involvement by x-ray. No other illnesses developed in either group of animals.
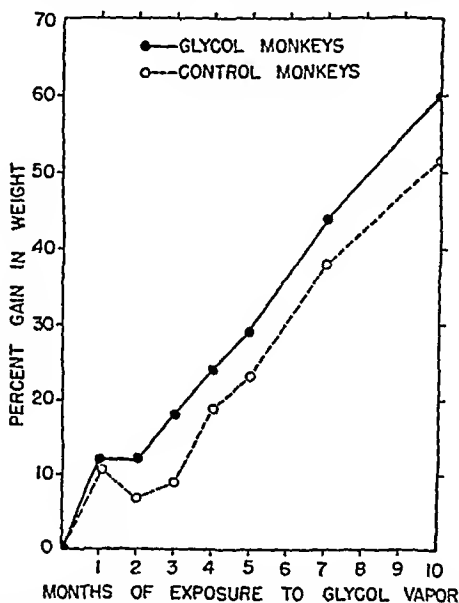
FIG. 8. COMPARATIVE GROWTH RATES OF MONKEYS KEPT CONTINUOUSLY IN AN ATMOSPHERE OF TRIETHYLENE GLYCOL VAPOR MAINTAINED BELOW THE SATURATION LEVEL (TEST NO. 2), AND A GROUP OF CONTROLS

Average initial weight of the seven monkeys in glycol group 2516 grams. The eight control animals averaged 2630 grams.

The growth rates charted in figure 8 show a progressive and satisfactory gain in weight of both test and control monkeys. After the first month the animals in the glycol showed a slight but consistently greater weight gain than did the controls. This result gives some support to the inference that the less rapid growth of the monkeys in the first triethylene glycol experiment was due to a diminished food intake.

In contrast to the course of the blood counts in the previous triethylene glycol experiment (first test) the numbers of red blood cells and amount of hemoglobin increased during the ten months of the study—about equally in both glycol-exposed and control animals (table 13, Test no. 2). None of the monkeys in the

glycol atmosphere showed any discoloration of the skin of the face or any change in the color or texture of the ears. They were all lively, exhibited glossy coats and gained weight about equally well with the exception of one of the test animals which showed a relatively small increase in weight.

Autopsy. Since this second experiment with triethylene glycol was conducted primarily for observations on weight-gain and skin effects, it was not considered necessary to do post-mortem examinations in all the monkeys in view of the completely negative findings in respect to any deleterious effect of the glycol in animals of the first test group. Three animals were sacrificed, two from the glycol group and one control. The internal organs were entirely normal with the exception of one of the test animals which exhibited a localized area of chronic gastritis at the pyloric end of the stomach. This was the single monkey which had shown an unsatisfactory weight gain from the beginning of the experiment.

In summarizing the above described tests on the toxicity of triethylene glycol for monkeys and rats it may be stated that among the large number of animals studied no pathological changes, ascribable to effect of the glycol were detected. The only disturbances which could be related to the presence of triethylene glycol vapor in the air were drying of the skin and slight interference with nutrition. That these two effects were due to an excess of glycol vapor (fog) was indicated by their absence in the second experiment in which the concentration of glycol was kept below saturation by means of the glycostat.

DISCUSSION

Two studies on the prolonged administration of propylene glycol have been reported, one by Seidenfelt and Hanzlik (7) the other by Morris, Nelson and Calvery (22). The animals tested—rats—either drank water containing 10 per cent propylene glycol or ingested the glycol incorporated in the food in quantities of 2.5 to 4.9 per cent of the total food weight. In the latter experiment the animals showed no ill effects after two years on such a regime. The quantities of glycol administered to these animals daily was several hundred times the amounts estimated to have been inhaled by the rats in our study. The only new information contributed by the present investigation of the toxicity of propylene glycol is the demonstration that the continuous residence of monkeys and rats for a year or more in an atmosphere supersaturated with the vapor of this glycol was without deleterious effect on the lungs or functional activity of the body as a whole. In fact the animals in the glycol atmosphere seemed to thrive somewhat better than the control groups as judged by weight gain, and increase in red blood cells and hemoglobin content.

On the other hand there are no data available on long term toxicity studies with triethylene glycol. Tests carried out previously have all been of an acute nature except those of Lauter and Vler (9). These authors found that young rats drinking water containing 3 per cent triethylene glycol showed no ill effect after 30 days. Adult rats given water containing 5 per cent of the glycol died—although the young rats survived. While the maximum concentration of glycol in

the water of the present experiment on oral toxicity was only a little over 2 per cent, the administration was continued for 13 months without detectable disturbance of the rats' physiology. Our monkeys ingested in proportion to their weight only one seventh the amount taken by the rats on the maximum dosage, but even this was approximately 100 times the quantity they could possibly take into the body through inhalation.

Since the absorption of triethylene glycol from inhalation of atmospheres containing this vapor represents such a minute fraction (less than 1/1000th of the toxic dose for rats) and since the experiments showed no evidence of either generalized pulmonary irritation, which one would expect from a toxic vapor, or of more localized disturbances, if glycol accumulated in the lungs, the particular value of the present investigation lies in the demonstration of the tolerance of these animals for prolonged exposure to glycol-containing atmospheres. Such information is of particular value in the case of monkeys since the responses of this animal to changes in environmental conditions approach much more nearly those of the human being than do the reaction of the common laboratory animals.

The question may of course be raised concerning the relative toxicity of triethylene glycol for animals and men. Such information as we have indicates that the effects of glycols on the tissues is much the same for the different animal species. Geiling and Cannon's (23) study of diethylene glycol, following the fatalities from elixir sulphanilamide, showed that the fatal dose for rats, rabbits and dogs was within the same range as that for humans. It should be pointed out that triethylene glycol is much less toxic for animals than is diethylene glycol. The comparative toxicities of the various glycols have been extensively reviewed by Van Oettingen (24).

While the outcome of our studies provided a high degree of assurance that exposure of human beings to triethylene glycol vapor could be safely undertaken, clinical tests of the effectiveness of triethylene glycol for aerial disinfection and the control of air-borne infections have contributed direct evidence of the inocuousness of this vapor in the air. In the studies of Harris and Stokes (25) Hamburger and associates (26) (16) and Bigg and Jennings (27) in which groups of individuals were exposed for periods ranging from several weeks to over a month at a time to atmospheres either partially or completely saturated with triethylene glycol vapor, no untoward effects of the glycol were observed. Loosli and co-workers (28) were able to detect no disturbance of the skin or other organs of very young infants kept continuously for 5-6 months in an atmosphere containing bactericidal concentrations of this glycol. Other observations (not reported in the literature) on large groups of industrial workers exposed during working hours to atmospheres partially saturated with triethylene glycol for many years have not recorded a single instance of ill effects attributable to this glycol vapor.

SUMMARY

With a view to determining the safety of employing the vapors of propylene glycol and triethylene glycol in atmospheres inhabited by human beings, monkeys and rats were exposed continuously to high concentrations of these vapors for

periods of 12 to 18 months. Equal numbers of control animals were maintained under physically similar conditions. Long term tests of the effects on ingesting triethylene glycol were also carried out. The doses administered represented 50 to 700 times the amount of glycol the animal could absorb by breathing air saturated with the glycol.

Comparative observations on the growth rates, blood counts, urine examinations, kidney function tests, fertility and general condition of the test and control groups, exhibited no essential differences between them with the exception that the rats in the glycol atmospheres exhibited consistently higher weight gains. Some drying of the skin of the monkeys' faces occurred after several months continuous exposure to a heavy fog of triethylene glycol. However, when the vapor concentration was maintained just below saturation by means of the glycostat this effect did not occur.

Examination at autopsy likewise failed to reveal any differences between the animals kept in glycolized air and those living in the ordinary room atmosphere. Extensive histological study of the lungs was made to ascertain whether the glycol had produced any generalized or local irritation. None was found. The kidneys, liver, spleen and bone marrow also were normal.

The results of these experiments in conjunction with the absence of any observed ill effects in patients exposed to both triethylene glycol and propylene glycol vapors for months at a time, provide assurance that air containing these vapors in amounts up to the saturation point is completely harmless.

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THE EFFECT OF DI-ISOPROPYL FLUOROPHOSPHATE ON NEUROMUSCULAR TRANSMISSION[1]

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The principal pharmacologic actions of di-isopropyl fluorophosphate (DFP) have been attributed to its ability to inactivate cholinesterase, (1, 2). The acute administration of DFP resulted in fasciculations and weakness of skeletal muscle in addition to parasympathomimetic effects. Modell et al. (2) noted that large doses of DFP administered intravenously to atropinized cats resulted in the development of profound muscular weakness which lasted for 1 to 2 weeks or longer. The gastrocnemii of such poisoned cats were unable to sustain a tetanus induced by nerve stimulation with an inductorium. Koelle and Gilman (3) reported the development of fasciculations of the skeletal muscles and hind leg weakness in dogs chronically poisoned with DFP. Harvey et al. (4) have studied the effect of DFP on neuromuscular transmission in normal and myasthenic man. The intra-arterial injection of DFP in normal man caused numerous spontaneous fasciculations and a pronounced weakness of muscles in the injected region. Electromyograms showed the second of 2 successive stimuli to be reduced in amplitude. In contrast, patients with myasthenia gravis showed a localized increase in muscle strength and a return to normal of the usual electromyographic defect. Riker and Wescoe (5) reported that the close intra-arterial injection of DFP in the cat gastrocnemius preparation caused unorganized contractions after a latent period of 2 to 5 minutes.

The present report is concerned with the effect of DFP on neuromuscular transmission in the intact cat. The effects of DFP were compared with those of 2 other anticholinesterases, prostigmine and physostigmine.

METHODS. Adult cats were used, weighing between 2.7 and 4.5 kg. A transection of the spinal cord at level L-1 was performed on all animals under preliminary ether anesthesia. The gastrocnemius muscle was freed by elevating the Achilles tendon with the posterior portion of the calcaneum. Blood supply to the muscle was carefully preserved, while other branches of the tibial vessels were ligated. Utilizing a holder described by Wolff and Cattell (6), the lower end of the femur was fixed by means of a steel pin and the tendon was wired vertically to a heavy isometric lever. The popliteal artery was exposed. Intra-arterial injections were made with a ⌗26 needle, during which time the artery was occluded from above. All doses were dissolved in a volume of 0.1 cc./kg. of distilled water. The intact sciatic nerve was stimulated with enclosed silver electrodes which were fixed in situ. Maximal break shocks were delivered from an inductorium by an interruptor, at a constant frequency of 1 in 12 seconds. Drying of the muscle was retarded by the repeated application of mineral oil to its surface.

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similar sensitization to the effects of acetylcholine by DFP has been noted on the blood pressure of the cat (2) and on the response of the frog rectus muscle (7).

The effect of DFP on curarized muscle. Figure 3 shows a typical response of the curarized gastrocnemius to the intra-arterial injection of DFP. A gradual restoration of the response to single nerve shocks occurs. The subsequent intra-arterial injection of prostigmine causes a further increase in the muscle response to nerve shocks. The effect of DFP in antagonizing curare is much less striking than that from prostigmine. In addition, fasciculations are not seen when DFP

Fig. 3. Spinal cat 3.0 kg. Isometric recording of gastrocnemius muscle. Stimulation of sciatic nerve by maximal break shocks 1 in 12 seconds. Intra-arterial injections: at 11:06 d-tubocurare 0.1 mg./kg.; at 11:12 DFP 200 μg./kg.; and at 11:19 prostigmine 10 μg./kg.

A   B

Fig. 4. Spinal cat 3.3 kg. Isometric recording of gastrocnemius muscle. Stimulation of sciatic nerve by maximal break shocks 1 in 12 seconds. Intra-arterial injections: at 3:47 DFP 100 μg./kg.; at 4:02 prostigmine 5 μg./kg.; and at 4:45 acetylcholine 0.5 μg./kg.

is injected into the partially curarized muscle but are evident following a subsequent injection of prostigmine.

The effect of DFP on the response to acetylcholine, prostigmine, and physostigmine during nerve stimulation. In normal muscle the intra-arterial injection of acetylcholine between stimuli results in a contractile response and the following nerve shock may evoke a slightly diminished or augmented response. There is no prolonged depression of transmission. After the intra-arterial injection of 100 to 200 μg./kg. of DFP, the subsequent injection of very small amounts of acetylcholine (0.1–1.0 μg./kg.) results in a depression of the response to nerve shocks. The duration of this depression varies with the dose of acetylcholine; after 10 μg./kg. it may persist for as long as 30 minutes (fig. 1). After smaller doses

RESULTS. Each of the following observations was confirmed by at least 3 experiments:

The effect of DFP on normal muscle. The intra-arterial injection of DFP during stimulation by maximal break shocks results in an increase in muscle tension developed by succeeding stimuli (fig. 1). With doses of 20 μg./kg. the onset of this potentiation is slow and the increase less than maximal. Larger doses usually cause an almost immediate effect and the potentiation is maximal after 100 μg./kg. The increase in tension may be as great as 100 per cent of the control and may last for 30 minutes or more. Amounts larger than 100 μg./kg.
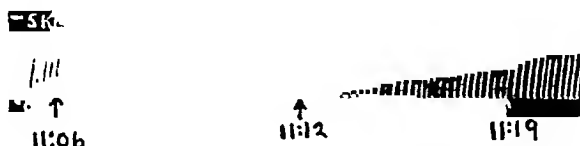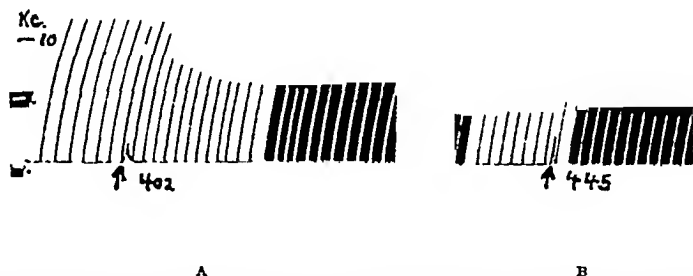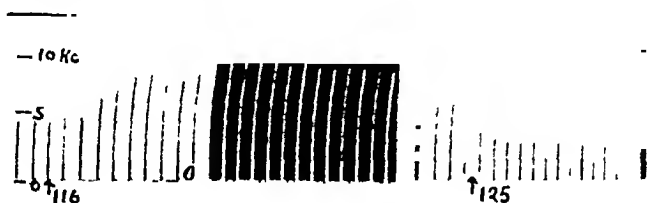

FIG. 1. Spinal cat 4.1 kg. Isometric recording of gastrocnemius muscle. *Stimulation of sciatic nerve by maximal break shocks 1 in 12 seconds.* Intra-arterial injections: at 1:16 DFP 100 μg /kg.; at 1:25 acetylcholine 10 μg./kg.
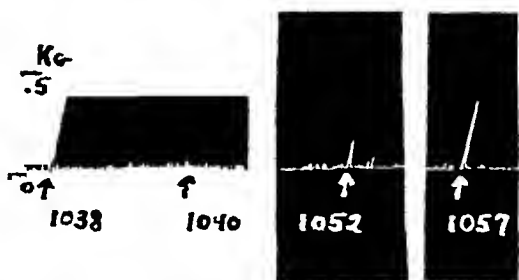
FIG. 2. Spinal cat 2 9 kg. Responses to intra-arterial injections. Light isometric lever. At 10:38 acetylcholine 1 μg /kg ; at 10:40 acetylcholine 0.5 μg./kg. ; at 10:46 DFP 100 μg./kg.; at 10:52 acetylcholine 0.1 μg /kg ; and at 10:57 acetylcholine 0 5 μg /kg.

cause a slight and gradual depression of the muscle response, but almost complete blocking of the nerve impulse at this frequency is not seen until amounts as high as 1 mg./kg. are given. *Potentiation of the muscle response to single shocks can also be obtained after the intravenous administration of DFP.* By this route doses as high as 3 mg./kg. are necessary for the production of maximal potentiation.

The effect of DFP upon the response to the close intra-arterial injection of acetylcholine is seen in figure 2. Prior to the injection of DFP the muscle failed to respond to doses of acetylcholine less than 1 μg./kg. Following 200 μg./kg. of DFP it responded to amounts of acetylcholine as small as 0.1 μg./kg. A

choline by a mechanism other than cholinesterase inhibition.   Miquel has shown that the frog rectus muscle, which has been soaked in a concentration of DFP sufficient to destroy cholinesterase activity, manifests a further sensitization to acetylcholine after exposure to physostigmine (7).   The response of the cat gastrocnemius to single nerve shocks is not further potentiated by physostigmine after a maximal increase in tension has been obtained with DFP.

It has been noted that the close intra-arterial injection of acetylcholine, after the administration of physostigmine, results in a depression of the muscular response to nerve stimulation (8).   Acetylcholine has a similar action after the intra-arterial injection of 100 to 200 μg./kg. of DFP.   If, however, the potentiation to single shocks resulting from DFP is due to the accumulation of acetylcholine, it is reasonable to expect an increased response to single shocks following an appropriate dose of acetylcholine.   This effect is noted after smaller amounts of DFP.

It should be emphasized that the experimental conditions are designed to show potentiation effects.   An increased rate of stimulation would show neuromuscular block when this potentiation effect is maximal at slower rates of stimulation. Thus a spinal animal, given large amounts of DFP intravenously, showed marked potentiation of the gastrocnemius response and yet became too weak to support its head.

The failure of the muscle to maintain a tetanus after DFP which was noted by Modell et al. occurred in cats which had extremely low blood pressures.   In the present study the blood pressure was determined by cannulation in a number of cats at the termination of the experiment.   In all cases the mean arterial pressure was above 100 mm. Hg.

The depressant effects of physostigmine and prostigmine after the administration of DFP may be explained in 2 ways: first, that a further inhibition of cholinesterase occurs, allowing liberated acetylcholine to accumulate in paralytic amounts; or secondly, that these agents have a direct nicotinic action in addition to their anti-esterase properties.   It has been shown that prostigmine, after large doses of DFP, renders the muscle refractory to injected acetylcholine (5).   Small amounts of prostigmine (5μg./kg.) when injected after 100 μg./kg. of DFP also cause a marked depression of the response to single nerve shocks, while repetition of the DFP dose may be without further effect.   This effect of prostigmine is in accord with the concept of a direct action on the effector organ.

The intra-arterial injection of physostigmine after DFP results in a slight depression of the response to single nerve shocks.   This effect is elicited by 20 to 40 μg./kg. of physostigmine; to obtain an equivalent depression the further injection of 100 to 200 μg./kg. of DFP would be required.   This ratio of dosage is similar to that necessary for maximal potentiation and can be attributed to the need for a critical degree of inactivation of cholinesterase to produce these effects. This suggests that physostigmine is a more potent inhibitor of cat muscle cholinesterase or that DFP is less capable of penetrating to the motor nerve endings.   In chronically denervated mammalian muscle no contractile response

of DFP (30–100 μg./kg.) the injection of 0.1 to 1.0 μg./kg. of acetylcholine causes an increase in the tension developed by succeeding stimuli (fig. 4 B). However, larger doses of acetylcholine cause depression under these circumstances.

After the intra-arterial injection of 100 to 200 μg./kg. of DFP, the muscle becomes extremely sensitive to prostigmine. Doses of 5 μg./kg. of prostigmine injected intra-arterially produce a depression like that seen after acetylcholine following similar doses of DFP (fig. 4 A). Larger amounts of prostigmine result in a refractory state from which the muscle often fails to recover.

The intra-arterial injection of 20 μg./kg. of physostigmine usually produces a maximal potentiation of the muscle response to nerve shocks. If, however, a maximal potentiation of the contractile response is produced by DFP, the subsequent intra-arterial injection of this dose of physostigmine causes a slight depression, usually lasting about 30 minutes (fig. 5). In contrast, the depression resulting from a further dose of DFP is not reversible.

The depression of the contractile response of the muscle to single nerve shocks
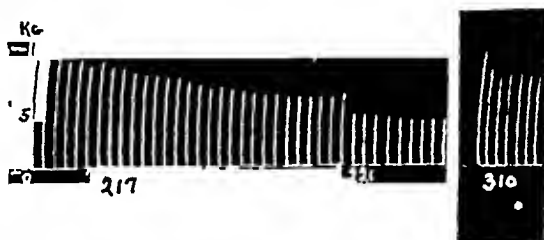
FIG. 5 Spinal cat 4 2 kg Isometric recording of gastrocnemius muscle. Stimulation of sciatic nerve by maximal break shocks 1 in 12 seconds Intra-arterial injections: at 2:03 DFP 100 μg /kg.; at 2:07 DFP 100 μg./kg ; at 2:17 physostigmine 20 μg /kg.; and at 2:20 physostigmine 20 μg./kg.

following excess amounts of DFP, physostigmine, and prostigmine is due to an action at the neuromuscular junction. This is evidenced by a much greater response of the muscle when stimulated directly than when stimulated via the nerve duing such depression. In addition, the intra-arterial injection of 1 mg./kg. of DFP is without effect on the response of the directly stimulated, completely curarized muscle.

DISCUSSION. The potentiation of the muscular response to maximal single nerve stimuli following the intra-arterial injection of DFP is similar to that seen after physostigmine. The latter was shown by Brown, Dale, and Feldberg to be due to the development of a partial tetanus (8). This presumably is caused by the accumulation of acetylcholine at the motor nerve endings, due to inhibition of cholinesterase. Bacq and Brown studied a variety of anti-cholinesterases and found that their ability to potentiate the muscle response to single nerve shocks paralleled their ability to inhibit cholinesterase (9). Certain recent evidence suggests that physostigmine may sensitize muscle to acetyl-

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is elicited from the injection of DFP or physostigmine (10), while the injection of acetylcholine or prostigmine is followed by a typical contracture (5). Unlike prostigmine, the evidence for a direct action of physostigmine on mammalian muscle is inconclusive. The fact that the prolonged muscular weakness following DFP can be prevented by the previous administration of small doses of physostigmine (11) makes it probable that this effect of DFP is due to its ability to inhibit cholinesterase irreversibly rather than to some other action.

Like prostigmine and physostigmine, DFP reverses the depression of neuromuscular transmission caused by curare. These agents may act by increasing the amount of acetylcholine at the end plate, thus exceeding the raised threshold caused by curare. Brown, Dale, and Felberg noted that after partial curarization of the cat gastrocnemius, the close intra-arterial injection of acetylcholine causes an increased response of the muscle to the succeeding few nerve shocks. DFP is much less effective than prostigmine in counteracting curare depression of the muscle. The intra-arterial injection of DFP into the partially curarized muscle does not produce fasciculations, whereas prostigmine does. These findings suggest that prostigmine is effective in antagonizing curare by a mechanism in addition to its inhibitory action on cholinesterase.

The configuration and duration of the contractile response to the intra-arterial injection of acetylcholine (5), or to a single nerve shock is not altered in the absence of cholinesterase. However, the maintenance of responsiveness of the muscle to these two types of stimulation depends on the integrity of the cholinesterase at the end plate. In such a role this enzyme may be said to be essential to junctional transmission at motor nerve endings.

SUMMARY

The effect of the intra-arterial injection of DFP and other cholinergic agents on the response of the cat gastrocnemius to maximal single nerve shocks is described.

1. DFP causes a potentiation like that seen following physostigmine.

2. DFP sensitizes the muscle to the close intra-arterial injection of acetylcholine.

3. DFP is less effective in antagonizing the depression of neuromuscular function caused by curare than is prostigmine.

4. Additional evidence is advanced for a direct action of prostigmine on skeletal muscle. A comparative study has been made of the actions of DFP, prostigmine, and physostigmine.

5. In relation to these data, the role of cholinesterase in neuromuscular transmission is discussed.

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Mode of Action. In view of the fact that sulfaquinoxaline was found capable of producing hypoprothrombinemia within twenty-four hours in rats or dogs on a complete diet, an action shown by no other sulfonamide, it became of interest to inquire into the mode of acton of this peculiar drug.

Studies *in vitro* failed to show evidence of anticoagulent action. Thus no change in the prothrombin activity of either oxalated rat's blood or dog's blood was observed when such blood was incubated aseptically at 37°C. for periods up to twenty-four hours, in the presence of 30 or 60 mg. of sulfaquinoxaline per 100 cc. of blood. This concentration of drug is considerably above that usually present in the blood of animals dosed orally with large quantities of sulfaquinoxaline.

Although it is well known that lowered prothrombin levels follow the use of hepatotoxic agents such as chloroform, phosphorous and tannic acid (11, 12, 13), histological examination of the livers of rats and dogs, rendered hypoprothrombinemic by the administration of sulfaquinoxaline, failed to reveal any abnormalities.

It has been suggested that the hypoprothrombinemia observed in animals after prolonged ingestion of sulfonamides in purified rations is due, in part at least, to a vitamin K deficiency resulting from decreased production of this vitamin because of inhibition of intestinal bacteria (5). Sulfaquinoxaline has been shown to reduce markedly the number of intestinal bacteria in rats and mice (14). However, since it appeared unlikely that hypoprothrombinemia would occur in so short a period as twenty-four hours, even in the total absence of intestinal bacteria, an experiment was performed to elucidate this point. The entire gastrointestinal tracts of 15 rats were removed surgically under ether anesthesia. The animals were maintained after operation by two injections daily of 10 cc. of an aqueous solution containing 5% glucose and 0.43% sodium chloride. Seven cc. of the solution were given intraperitoneally and 3 cc. were given subcutaneously. Six of the rats survived for twenty-four hours, 3 for forty-eight hours, 2 for seventy-two hours and 1 for one hundred twenty hours. Prothrombin levels, determined daily, were normal in all rats. This experiment indicates that the body stores of prothrombin and/or vitamin K are sufficient to maintain normal prothrombin concentrations in the blood of rats for several days even though no vitamin K is administered and no endogenous vitamin K is supplied through synthesis by intestinal flora.

A study was made to determine whether the hypoprothrombinemic action of sulfaquinoxaline could be attributed to one of the constituent portions of the molecule. Two quinoxaline compounds, amino quinoxaline and 2-methylquinoxaline, were given orally to rats for a period of two to three weeks, the former compound at a level of 1000 mg. per kg., the latter at 500 mg. per kg. Neither compound effected a significant change in the prothrombin level. Both sulfanilamide and sulfamerazine given in the diet at a level of 0.5% (equivalent to about 500 mg. per kg.) failed to produce hypoprothrombinemia within four weeks. These data suggest that the prompt hypoprothrombinemic action of

HYPOPROTHROMBINEMIA RESULTING FROM THE ADMINISTRATION OF SULFAQUINOXALINE

CHARLES W. MUSHETT AND ALBERT O. SEELER[1]

With the Technical Assistance of FRIEDA GLASS SCHREIBER

From the Merck Institute for Therapeutic Research, Rahway, N. J.

The property of inducing hypoprothrombinemia has been attributed to a number of drugs such as dicumarol (1), salicylates, including acetylsalicylic acid (2), quinine sulfate (3) and sulfonamides (4, 5, 6). Of particular interest is the fact that sulfonamides in general are effective in bringing about a reduction in the prothrombin level of blood only when fed to animals maintained on a highly purified diet. Furthermore the time required to bring about this change is relatively long. In a previous communication (7) we reported that 2-sulfanilamido quinoxaline (hereafter called sulfaquinoxaline), a heterocyclic sulfonamide synthesized by Weijlard, Tishler and Erickson (8), is capable of producing a rapid hypoprothrombinemia in dogs and rats receiving stock rations. Vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone) prevented this condition while menadione (2-methyl-1,4-naphthoquinone) was much less active in this respect. The present communication represents a further study of sulfaquinoxaline with particular reference to its mode of action in producing hypoprothrombinemia and to the relative activity of vitamin K compounds in controlling this condition.

Albino rats (Carworth Farms) weighing 150 to 250 grams and adult mongrel dogs weighing 8 to 12 kilograms were used in these experiments. All animals received a nutritionally adequate stock ration. The method of Campbell and coworkers (9) was followed for the determination of prothrombin in dogs, using 100% and 12.5% plasma. The micro method of Hoffman and Custer (10) was adapted for use in the rat. Pipettes of 0.1 cc. capacity, graduated in 0.01 cc. were employed. Several pieces of rubber tubing, of the type provided with ordinary blood pipettes, were fastened together and connected to the mouth piece of the pipette to provide greater ease in manipulation. A volume of 0.05 cc. of blood was taken from the rat's tail and discharged into a micro depression slide containing the thromboplastin preparation. A constant temperature of 38°C. for the reaction was maintained by fastening the micro slide to a hot water bottle placed in a cardboard box provided with projecting sides to prevent rapid cooling by air currents. After withdrawal of blood from the tail, collodion was applied to prevent further bleeding. By this method each rat could be tested repeatedly without producing anemia or death, frequent sequelae of methods involving cardiac puncture for removal of blood.

The drugs were introduced into the stomach of the rats by means of a blunt metal catheter. Dogs were dosed by stomach tube. The sulfaquinoxaline[2] was

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[2] The sulfaquinoxaline and derivatives of this compound were kindly supplied by Drs M. Tishler, F. J. Wolf, K. Pfister, III, R. Beutel and J R Stevens of the Merck Research Laboratories.

suspended in 10% gum acacia and the vitamin K compounds were dissolved in ethyl laurate.

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FIG 1. CORRELATION OF BLOOD LEVELS AND PROTHROMBIN TIMES IN DOGS
GIVEN SINGLE OR MULTIPLE ORAL DOSES OF SULFAQUINOXALINE

TABLE 1

Correlation of blood levels and prothrombin times in rats given a single oral dose of sulfa quinoxaline (5 rats per group)

TIME	GROUP I, 400 MG /KG.		GROUP II, 250 MG /KG.	
	Average blood level	Average prothrombin time	Average blood level	Average prothrombin time
hours	mg %	seconds	mg. %	seconds
0	0.0	20.0	0.0	16.0
6	8.4	17.8	7.5	21.4
13	8.7	23.4	7.2	20.6
22	12.4	45 6	10.0	43.8
46	9.4	108 4	8.6	61.8
74	7.1	130.2	5.9	36.2
106	5.1	11.6	2.8	11.6

sulfaquinoxaline cannot be attributed solely to either the sulfanilamido or the quinoxaline portion of the molecule, but is due to the combination of both.

The relationship between sulfaquinoxaline blood concentration and prothrombin time was investigated in dogs (fig. 1) and rats (table 1). Dogs given a single oral dose of 250 mg. per kg. showed a peak blood concentration of the drug twenty-four hours after treatment. The most prolonged prothrombin time, however, did not occur until the second day. When dogs were treated daily with 100 mg. per kg. the blood concentration rose gradually and tended to level off after a few days. The prothrombin time increased significantly after the second dose of drug. Failure of the plasma to clot within 30 minutes was observed on the fifth or sixth day. Death resulted from hemorrhage several days later.

Results obtained in rats given a single oral dose of the drug were similar to those in dogs. Following the administration of 250 mg. per kg. the highest blood concentrations were present at twenty-two hours and the most prolonged prothrombin times at forty-six or seventy-four hours. As the drug disappeared from the blood stream, the prothrombin time decreased to a point even lower than that initially recorded.

A group of 10 rats were given 3 daily doses of 200 mg. per kg. of sulfaquinoxaline. On the fourth day determinations were made of prothrombin time and plasma fibrinogen concentration. The fibrinogen level averaged 416 mg.% (235–635) compared to 239 mg.% (210–265) for 5 control rats. Six of the treated animals showed elevations in both prothrombin time and fibrinogen level, 2 in prothrombin time alone and 2 in fibrinogen level alone. Thus in rats treated with sulfaquinoxaline the decrease in plasma prothrombin concentration tends to be associated with an increase in the level of fibrinogen.

With prolonged administration of sulfaquinoxaline, in the absence of vitamin K therapy, hypoprothrombinemia of increasing severity occurred and caused or contributed to the death of the animal. In a few cases, however, spontaneous recoveries were observed in rats and dogs despite continuous dosing with this compound. In approximately 400 rats, 3 such recoveries were noted. Each of 2 dogs which were dosed daily by mouth with 25 mg. per kg. showed a peak prothrombin time (i.e., the lowest prothrombin level) on the twentieth day of test. By the thirty-second day the prothrombin time had returned to normal where it remained with slight fluctuations despite fifty-five additional days of drug treatment (fig. 2). In 1 of 4 dogs given 100 mg. per kg. orally per day a peak prothrombin time was reached on the twenty-first day and complete spontaneous recovery was observed by the fifty-sixth day. Two weeks additional dosing beyond this point caused no further change in the prothrombin level. No explanation for these spontaneous recoveries is evident, but several possibilities exist. As a result of prolonged exposure to sulfaquinoxaline, a resistant strain of bacteria may have arisen in the intestinal tract, a strain capable of producing large quantities of vitamin K. On the other hand, the continued presence of sulfaquinoxaline in the blood may have resulted in a compensatory increased output of prothrombin by the liver.

Comparison of Activity of Vitamin K Analogues. Several preliminary experiments had shown that vitamin K_1 was more active than menadione in preventing sulfaquinoxaline-induced hypoprothrombinemia when given orally in doses of

1 mg. per rat per day.   Menadione, in fact, appeared to be inactive at this level. In order to evaluate more precisely the relative activity of these two compounds, they were administered orally at different dose levels to rats receiving daily oral
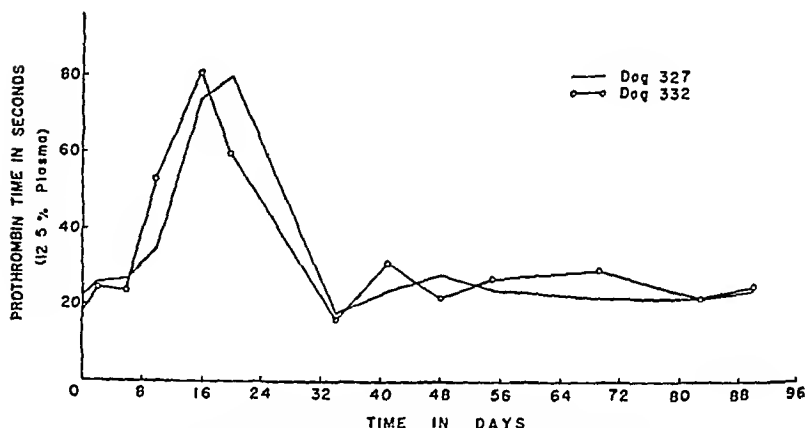
FIG 2. SPONTANEOUS REMISSION FROM SULFAQUINOXALINE
INDUCED HYPOPROTHROMBINEMIA
Both dogs received 25 mg per kg daily for eighty-seven days

TABLE 2

Comparative activity of vitamin K_1 and menadione in preventing sulfaquinoxaline-induced hypoprothrombinemia

NO OF RATS	SULFAQUINOXA-LINE, DAILY ORAL DOSE	K COMPOUND	DAILY ORAL DOSE PER RAT	TOTAL NUMBER OF RATS SHOWING HYPOPROTHROM BINEMIA AFTER	
				6 doses	16 doses
	mg per kg		*mg.*		
10	200	Vitamin K_1	10	0	0
10	200	Vitamin K_1	1	0	3
10	200	Vitamin K_1	0 1	7	9
10	200	Menadione	25	6	8
10	200	Menadione	10	10	10
10	200	Menadione	5	10	10
10	200	Menadione	1	9	10
10	200	None	—	9	10
10	None	None		0	0

doses of sulfaquinoxaline (200 mg. per kg.).   Groups of 10 rats each received 10, 1 or 0.1 mg. of vitamin K_1 ; 25, 10, 5, or 1 mg. of menadione, or no K therapy. The results are shown in table 2. On the basis of protection from hypopro-

thrombinemia and survival, 0.1 mg. of vitamin K_1 was slightly more effective that 10 mg. of menadione but less effective that 25 mg. of menadione. It would appear from these data that the anti-hypoprothrombinemic activity of vitamin K_1 is approximately 100–250 times that of menadione on a weight basis. Tested under comparable conditions, sodium 2-methyl-1,4-naphthoquinone disulfate was found to be more active than menadione, but less active than vitamin K_1. Diethyl phthalate proved to be entirely ineffective at a dose level of 5 mg.

Derivatives of Sulfaquinoxaline. Several derivatives of sulfaquinoxaline were tested also in rats for hypoprothrombinemic acivity. The 6(or 7)-methyl, 6(or 7)-chloro and N4-acetyl derivatives were effective when given orally in doses of 500–1000 mg. per kg. daily for a week, while the 6(or 7)-methoxy derivative failed to produce hypoprothrombinemia within this time at a dose level of 500 mg. per kg. It was of interest to note that transposition of the sulfanilamido group from the 2 to 6 position resulted in a complete loss of activity of sulfaquinoxaline.

DISCUSSION. Sulfaquinoxaline, like other sulfonamides (15), salicylates (2), dicumarol (1), and certain naphthoquinones (17) does not exert an anticoagulant effect *in vitro*. It would appear, for this reason, that its prothrombinopenic effect is not due to direct destruction or inactivation of prothrombin in the blood. The sulfonamides as a group presumably induce hypoprothrombinemia in an indirect manner by curtailing the synthesis of vitamin K through inhibition of the intestinal bacteria responsible for this synthesis. They are effective, however, only when the treated animals are maintained on purified diets which supply little or no exogenous vitamin K. Unlike the other sulfonamides, sulfaquinoxaline brings about a prothrombinopenia when given orally to dogs and rats maintained on stock rations. Furthermore, since the present study indicates that rats do not develop hypoprothrombinemia within several days after total removal of the gastro-intestinal tract, it would seem unreasonable to assume that the rapid hypoprothrombinemic effect of sulfaquinoxaline, which is manifested within twenty-four hours, is related to inhibition of intestinal bacteria.

It is well recognized that hepatic injury, through the use of chloroform and other hepatotoxic agents, will result in a lowered prothrombin concentration in the blood (11, 12, 13). A rapid decline in plasma prothrombin, detectable within a few hours, occurs after total hepatectomy (16). The absence of gross and histological changes in the livers of rats and dogs rendered hypoprothrombinemic by sulfaquinoxaline suggests that hepatic damage is not involved in the mode of action of this drug. It is possible, however, that hepatic dysfunction may have occurred even though no anatomic changes were evident. Both prothrombin and fibrinogen are decreased in the bleeding state due to chloroform-induced hepatic injury (12), whereas the decrease in prothrombin level of rats after sulfaquinoxaline administration tends to be accompanied by an increase in plasma fibrinogen.

Recently a new group of compounds, derivatives of 3-hydroxy-1,4-naphthoquinone, have been shown to possess hemorrhagic potency (17). It is postulated that they produce this effect by competing with vitamin K in the process of

prothrombin synthesis. Clausen and Jager (18) have suggested that salicylates and dicumarol, by virtue of their chemical relationship to vitamin K, may block the utilization of this vitamin in the manufacture of prothrombin. The counteraction of this phenomenon by very large doses of vitamin K they would attribute to a mass action effect. As pointed out, however, in a review of metabolite antagonists by Roblin (19), not all important antagonists are structurally related to the affected metabolite. The lack of chemical similarity of sulfaquinoxaline and the quinones would not appear to obviate the possibility that this sulfonamide acts as a vitamin K antagonist.

Vitamin K-like activity has been claimed for phthalic acid and derivatives such as diethyl phthalate by certain investigators (20) and disavowed by others (21, 22, 23). The present limited studies conducted with diethyl phthalate failed to show any such activity by this compound.

Numerous investigators have shown that the hypoprothrombinemia induced by dietary deficiency or by chemical substances, except in the presence of established hepatic damage, can be counteracted by vitamin K compounds. In the majority of these studies menadione (2-methyl-1,4-naphthoquinone) has been used. Although the potency of this compound has been compared with that of vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone) in vitamin K deficient chicks, few such comparisons have been conducted in mammals rendered hypoprothrombinemic by the administration of chemical agents. Using the chick assay for antihemorrhagic activity, Fieser, Tishler and Sampson (24) found menadione to be 3.3 times as potent as vitamin K_1 on a weight basis. Data of other investigators, cited by these authors, show ratios of 2.1:1 to 4:1 in favor of menadione. A relatively large dose of menadione is required to completely protect the rabbit against the anticoagulant action of dicumarol (25). Smith and coworkers (17) found menadione to be much less effective than vitamin K_1 in counteracting the hypoprothrombinemia induced in rats by 2-(3-cyclohexylpropyl)-3-hydroxy-1,4-naphthoquinone. In the present study vitamin K_1 was found to be 100–250 times as potent on a weight basis as menadione in sulfaquinoxaline-treated rats. Evidence suggesting that menadione may be relatively more active in the chick than in mammals is provided by the activity ratios for menadione to phthiocol (2-methyl-3-hydroxy-1,4-naphthoquinone) which have been reported as follows: in vitamin K deficient chicks, 1600:1 (24), in rats with ligated bile ducts, 500:1 (26), and in dicumarol-dosed rats, 2:1 (27). The explanation for this great difference in the relative activity of menadione and vitamin K_1 under different methods of test is not apparent. Perhaps menadione is more readily utilized than vitamin K_1 in the synthesis of prothrombin in the chick, while the reverse is true in mammals.

SUMMARY

The mode of action of sulfaquinoxaline in inducing a prompt hypoprothrombinemia does not appear to be related to anatomic changes in the liver or to inhibition of intestinal bacteria. Although this compound does not destroy or inactivate prothrombin *in vitro*, the possibility of such an action *in vivo* has not been excluded.

The rapid prothrombinopenic effect of sulfaquinoxaline cannot be attributed lone to either the sulfanilamido or the quinoxaline portion of the molecule, but is ue to the combination of both as present in this sulfonamide. Transposition of he sulfanilamido group from the 2 to the 6 position resulted in loss of this activity.

Vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone) was found to be 100–250 imes more effective, on a weight basis, than menadione (2-methyl-1,4-naphtho-quinone) in preventing the hypoprothrombinemia which results from the administration of sulfaquinoxaline.

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ERRATUM

in paper by Albert Wollenberger "Metabolic Action of the Cardiac Glycosides
Influence on Respiration of Heart Muscle and Brain Cortex", Journal of Ph
macology and Experimental Therapeutics, Vol. 91, No. 1, pp. 39–51 (Sept. 194

p. 39, 5th line from the bottom: read 0.6 mm. instead of 0.5

p. 43, line 4: read table 4 instead of table 5

p. 43, in heading of table 3: read oxygen uptake instead of respiration

p. 50, line 6 in item 6 of summary: read into the cell instead of in the cell

A COMPARISON OF RACEMIC AND LEVO GLYCERO-GUAIACOL ETHERS FOR ANESTHETIC AND OTHER ACTIONS

H. WARD SMITH

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Racemic glycero-guaiacol ether is sold as an expectorant under the proprietary name of "Resyl". Its activity in increasing the water content of respiratory tract fluid has been investigated by Connell, Johnson and Boyd (1) and by Perry and Boyd. (2).

The present study was undertaken in order to assess the toxicity of glycero-guaiacol ether and in preliminary experiments it was discovered to have anesthetic and emetic properties. The anesthetic properties were sufficiently favourable that some attempt to separate them from the emetic actions seemed worth while.

At the suggestion and through the kindness of Dr. H. O. L. Fischer[1] a supply of 1-glycero-guaiacol ether was made available so that a comparison of the anesthetic and emetic potencies of the levo and racemic materials could be made.

Two methods of assaying anesthetic potency which have a wide application are compared in the course of this study.

PRELIMINARY OBSERVATIONS. Racemic glycero-guaiacol ether is a white crystalline solid melting between 77.7 and 78.5°C. It is soluble to the extent of 5% in water at room temperature; the water must be warmed slightly to dissolve this amount rapidly. The material is bland enough to be taken by mouth in 0.5 gm. amounts, in which case a prolonged slightly bitter taste was the only observed sensation. The levo isomer has similar physical properties but has a higher melting point, 95.0–97.8°C., and is only soluble to the extent of 2% in water. Throughout these studies there was no indication that these materials were irritant when injected intraperitoneally in unanesthetised kittens or mice. Intravenous injection had no effect on the blood pressure or respiratory pattern of a lightly anesthetised cat.

Intraperitoneal injection of 0.5 to 1.0 gram/kgm. of racemic glycero-guaiacol ether in the mouse was followed by a brief period of hyperactivity, followed by a loss of corneal and conjunctival reflexes. At this stage, and for approximately an hour, with the 1.0 gram/kgm. dose, the mouse was completely relaxed; there was no withdrawal reflex on pinching the toes; respiration was slow, deep and regular. Recovery from this dose was complete within 10 minutes of the time of return of corneal reflex.

A similar sequence of events was noted in a cat and in this animal slow intravenous injection (4% aqueous solution of racemic material) caused little disturbance of blood pressure up to the time of death from a lethal dose of 1.2 gram/kgm. Up to the time of death, which was due to respiratory failure, the heart rate was uniform and satisfactory systolic and pulse pressures were maintained. In this animal the corneal reflex was abolished by 0.6 gram/kgm. but strong galvanic stimulation of a peripheral nerve (sciatic) caused a mild disturbance in the respiratory tracing, even when the dose was increased to 1.0 gram/kgm. Intravenous injection of racemic material caused vomiting and defecation in the dog in subanesthetic doses. Intraperitoneal injections in kittens in subanesthetic doses, caused salivation and vomiting.

ANESTHETIC POTENCY. The anesthetic potency of racemic and levo glyceroguaiacol ethers was compared using a technique similar to that outlined by Hunt, Fosbinder and Barlow (3), using duration of loss of reflexes as a measure of potency.

In these experiments male mice weighing between 15 and 24 grams were starved overnight and injected intraperitoneally with 0.04 cc./gram of solutions of either racemic or levo glycero-guaiacol ether which were adjusted to deliver 0.56, 0.75 or 1.0 gram/kgm. of mouse. Pinna twitch was elicited by means of a feather and the corneal reflex by means of the tip of fine wire blunted by a drop of solder.

Table 1 shows the average response in terms of duration of loss of the various reflexes studied at the dose levels used in the determination of anesthetic potency. Each of the figures is an average obtained from the data of eight mice. Table 1 illustrates that the relationships of dose to response are similar for the three reflexes studied; indeed, when these figures are plotted the lines relating log dose to response are parallel. The data for duration of loss of pinna twitch reflex were used for statistical comparison of the relative potencies of the racemic and levo materials.

The responses obtained in the comparison of a sample of racemic material (R) with the levo isomer (L) are analysed in table 2 to demonstrate the validity of an assay conducted in this manner. The method and nomenclature of Bliss (4, 5) are used. Table 2 demonstrates that there was no significant difference in the potency of the samples studied, that the dose-response curve had a steep slope, that the slope for each material was similar, and that there was no appreciable departure from linearity of both lines, or curvature between lines relating log dose to duration of loss of pinna twitch reflex. Thus the conditions necessary for a valid assay have been met by this arrangement.

The potency of the racemic material was found to be 107% of the levo isomer with a range (± 2 Se) of 94-121% (P = 0.05).

A comparison of the anesthetic potencies of these materials was also made using the revolving wire screen, as described by Young (6). Male mice, 17 to 21 grams in weight, fasted for 12 hours, were injected intraperitoneally with 0.70 cc. of the material in question. The mice were placed on the revolving drum immediately after injection and rotated for 15 minutes at a speed of

$1\frac{1}{4}$ r.p.m. Counts were made of the number of animals that developed ataxia to the degree that they were unable to maintain a footing on the inclined (60°) revolving screen. The ataxia developed in 2-5 minutes in the doses employed and in 10 minutes all the animals that developed this degree of ataxia had dropped off the screen. The counts were treated by the method described by De Beer (7, 8). As in the previous method, the potencies of the racemic and levo materials were not significantly different; the racemic material was 109% of the

TABLE 1

Duration of loss of reflex

DOSE OF GLYCERO GUAIACOL ETHER GMS./KG.	0 56	0 75	1.00
Reflex	min	min.	min
Pinna Twitch .	40	79	128
Righting	25	64	116
Corneal		9	64

TABLE 2

SOURCE OF VARIANCE	FACTORIAL COEF. (x) FOR DOSE						NS(x)²	−SxY_p	VARI- ANCE	VARI- ANCE RATIO F
	Racemic			Levo						
	R1	R2	R3	L1	L2	L3				
Difference between samples	−1	−1	−1	+1	+1	+1	24	92	352	0.64
Slope of dosage response curve	−1	0	+1	−1	0	+1	16	705	31,064	56.0*
Departure from parallelism	+1	0	−1	−1	0	+1	16	−17	18	
Curvature of combined curves	+1	−2	+1	+1	−2	+1	48	−71	105	
Opposite curvature of separate curves	−1	+2	−1	+1	−2	+1	48	−193	572	1.04
Total responses for each dose (Yp)	514	364	153	507	269	163				

* Significant.

levo isomer with a range (±2 Se) of 96 to 122%. The E.D.$_{50}$ for the racemic material was 0.28 ± 0.02 gram/kgm. and for the levo isomer was 0.31 ± 0.01 gram/kgm.

The revolving screen method gave a similar result with a similar degree of precision, using 40 animals as the previous method, which was based on 24 animals. However, the revolving screen may be adapted to routine assay and presents advantages both from the point of view of economy of time spent in conducting the assay, calculation of results, and lack of subjective error in determination of the response. These advantages still hold when the calculation is conducted in the more rigorous manner of Bliss (9, 10) or Fisher (11).

LETHAL DOSE. The lethal dose was obtained in mice using the method of Miller (10) and calculated according to the graphic method of De Beer (8). As before, male mice weighing from 17 to 21 grams were used. These were starved overnight and injected intraperitoneally with 0.04 cc./gram of body weight of the material in question.

The lethal potency of the racemic material was found to be 100% with a range (± 2 Se) of 85 to 117% of the levo isomer. The L.D.$_{50}$ was 1.29 ± 0.16 gram/kgm. for both materials.

EMETIC DOSE. The emetic dose was determined in kittens by the procedure of injecting, intraperitoneally, an initial amount of approximately $\frac{2}{3}$ of the emetic dose as approximated from preliminary tests on two kittens. This dose was followed by successive injections of approximately $\frac{1}{15}$ of the emetic dose, every 5 or 10 minutes until emesis occurred.

The racemic material was given to one kitten on a total of five occasions, when the provocative doses followed the initial dose by 10 minute intervals. The emetic dose obtained from these 5 results was 0.36 ± 0.12 gram/ kgm.

In addition, a comparison of the emetic effect of levo and racemic materials was attempted. The levo isomer produced emesis less often than the racemic material. When four kittens were given the levo isomer for the first time, none of them showed signs of emesis. While salivation was marked, these kittens passed through the emetic stage to that of general depression. On the other hand, only one out of five kittens failed to show emesis when given racemic material for the first time.

All the animals showed emesis when given either material on a second occasion and the emetic doses obtained when these materials were given to three kittens the second time were as follows:

Racemic. . . . .   0 40 ± 0 10 gram/kgm.
Levo   0 33 ± 0.14 gram/kgm.

The above values are the mean and standard deviation of the three results on each material when only the second presentation of material was considered.

Marked salivation was often noted immediately after the initial dose in these kittens. The degree of this effect appeared quite variable and on several occasions obvious "drooling" was not noted.

SUMMARY AND CONCLUSIONS

1. The levo isomer of glycero-guaiacol ether was found to have the same anesthetic potency by the several methods used and the same lethal potency as the racemic material. Thus these actions of the glycero-guaiacol ethers do not depend on the stereo-configuration of the asymmetric carbon atom located in the glyceric portion of the molecule.

2. The emetic dose of levo glycero-guaiacol ether appeared to be of the same order of magnitude as that of the racemic material. However, emesis did not occur so often with the levo isomer. Thus the emetic action may be modified to some extent by the stereo-configuration.

3. The emetic dose of the racemic material (0.36 gram/kgm. kitten) and the lethal dose (1.29 gram/kgm. mouse) are much higher than the dose required to increase the respiratory tract fluid (0.01 gram/kgm. rabbit (1)).

4. Glycero-guaiacol ether has favourable anesthetic properties if the emetic effect could be elimiated. Substitution of one of the homologues of guaiacol in such a configuration might prove fruitful in this regard.

5. In the course of this study two methods of assay of anesthetic potency have been used. The revolving screen method gave a similar degree of precision, $\pm 15\%$ using 40 animals, as did the technique based on length of time of loss of various reflexes, when 24 animals were used. However, the revolving screen method is more readily adapted to routine assay and presents advantages with respect to decreased subjective error, and increased economy of time and personnel.

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SOME EFFECTS OF 1-METHYL-4-PHENYL ETHYL ISONIPECOTATE (DEMEROL) AND 6-DIMETHYLAMINO-4,4,-DIPHENYL-3-HEPTANONE (AMIDONE[1]) UPON THE METABOLISM OF RAT BRAIN TISSUE IN VITRO[2]

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The similar *in vivo* effects of morphine, demerol, and amidone prompted the following studies on rat brain metabolism to correlate, if possible, *in vivo* activity with *in vitro* effects on mechanisms of biological oxidation. These compounds possess entirely different chemical structures but pharmacological studies indicate that demerol (1) and amidone (2) *in vivo* are morphine-like in action.

Quastel and others (3, 4, 5) have investigated the actions of anesthetics and hypnotics on brain metabolism and have been able to show that parallels exist between *in vivo* and *in vitro* activity. For example, the ability of a series of barbiturates to inhibit the oxygen uptake of brain tissue respiring in glucose-Ringers solution is roughly proportional to their hypnotic potency (3). On the basis of metabolic studies on brain tissue preparations, Michaelis and Quastel (6) proposed that narcotics act by inhibiting the oxidation by brain of carbohydrate. According to Grieg (7) the inhibition is due to the binding of reduced flavoprotein with cytochrome b or other intermediate, thus interrupting the "main line of biological oxidation".

Morphine apparently exerts a different action upon metabolic processes. We have substantiated the results of Seevers and Shideman who showed that 0.12% morphine has no effect on the oxygen uptake of rat cerebral tissue respiring in glucose-Ringers. Presumably morphine does not affect the mechanisms of biological oxidation in a manner similar to other narcotics which have been studied.

The following experiments suggest that amidone and demerol likewise do not conform to Quastel's theory of narcosis although they are capable of exerting profound effects on brain tissue metabolism.

METHODS. Oxygen consumption and anaerobic glycolysis were measured by the direct method of Warburg (8, 9) at 37.2° C. Adult albino rats of the Slonaker-Wistar strain were sacrificed without previous preparation or medication. The brains were removed and placed in a cold box (10) as quickly as possible. Cerebral cortex slices were cut with a razor and template (11). Cerebral cellular homogenates were prepared in a Potter homogenizer (12). The tissues were suspended in a modified Krebs-Ringer solution containing 0.2% glucose or other substrate. In order to obtain a medium corresponding more nearly

to the composition of interstitial fluid, the amounts of potassium and calcium specified in Krebs' formula (9) were reduced from 5.1 to 4.0 and 5.4 to 3.2 millequivalents per liter respectively. Phosphate buffer was used for oxygen uptake measurements; bicarbonate buffer for anerobic glycolysis studies. The gas phase was oxygen for slices, air for homogenates and a 95% nitrogen 5% carbon dioxide mixture for anaerobic glycolysis studies. The last traces of oxygen were removed from the anerobic glycolysis vessels by means of a small piece of yellow phosphorus placed in the center well. Carbon dioxide was absorbed from the aerobic vessels by 0.1 ml. of 10% potassium hydroxide in the center well. The drugs (as hydrochlorides) were made up in Ringer's solution and added to the tissue from the sidearm after a control period of 60 or 90 minutes, each vessel thus serving as its own control. In addition, controls were run for the entire experimental period.

EFFECTS ON GLUCOSE OXIDATION. *Morphine.* Seevers and Shideman (13) found that 0.12% (0.0016M) morphine sulfate did not alter the respiration of brain slices in glucose-Ringers. Our measurements (figure 1) indicate that even higher concentrations are without effect. 0.005 and 0.01 M morphine hydrochloride failed to depress the oxygen uptake of brain slices. If uniform distribution were to be assumed, these concentrations would correspond to in vitro doses of 1.9 and 3.8 grams per kilogram, respectively.

Demerol. The effect of a series of concentrations of demerol upon the oxygen uptake of rat brain cortex slices is illustrated in figure 1. Considering the control Q_{0_2}'s (wet weight) for each concentration as 100%, the rate of oxygen uptake during the 90 minute period following addition of the drug is expressed as per cent of the control Q_{0_2}. Unlike morphine, demerol inhibits brain slice respiration but only at concentrations which correspond to very high in vitro doses. The lowest concentration of demerol to show inhibition of oxygen uptake (0.005M) is equivalent to 1.9 grams per kilogram, assuming uniform distribution.

Amidone. The action of various concentrations of amidone on the respiration of rat brain cortex slices is shown in Figure 1. The ordinate is expressed in per cent as described for demerol. This substance stimulates brain slice respiration at concentrations between 0.00016 and 0.0006 M but is a powerful inhibitor at concentrations above 0.001M. Apparently amidone exerts a dual effect, best shown at a concentration of 0.0006 M, resulting in a stimulation of respiration followed by depression. Amidone is a much more powerful inhibitor of tissue respiration than is demerol and its ability to stimulate oxygen uptake is not shared by either morphine or demerol. However, its effects are produced by concentrations much higher than are necessary to produce analgesia in vitro, assuming uniform distribution. The concentration of amidone which stimulates oxygen uptake over the entire experimental period is equivalent to 55 milligrams per kilogram (0.00016 M) and 0.001 M is equivalent to 345 milligrams per kilogram.

SITE of ACTION. Demerol and amidone in concentrations capable of inhibiting the oxidation of glucose do not inhibit the oxidation of succinate. This would indicate that these agents do not depress the cytochrome-cytochrome oxidase system (7).

The inhibition of cerebral homogenate respiration produced by demerol and amidone is not reversed by 6×10^{-4} M methylene blue in contrast to the complete reversal by methylene blue of sodium pentobarbital inhibition of similar preparations. Since methylene blue can serve as a hydrogen carrier between dehydrogenases and oxygen (7), demerol and amidone apparently inhibit the dehydrogenases involved in carbohydrate oxidation.

Anaerobic glycolysis of cerebral homogenate preparations is inhibited both by demerol and amidone as illustrated in table 1. The concentrations used were those which strongly inhibited brain slice oxygen uptake. Since these drugs decrease the anaerobic production of lactic acid, an action must occur before the separation of anaerobic and aerobic cabohydrate metabolism (14). This ability to inhibit anaerobic glycolysis is not shared by such narcotics as chloretone (6) or propazone (15). Because of the inhibition of anaerobic glycolysis it might be inferred that the principal action of demerol and amidone is to limit the supply of intermediary metabolites at a point previous to the separation of anaerobic and aerobic carbohydrate metabolism. However, this hypothesis is untenable because the oxidation of d-lactate and pyruvate is also inhibited by demerol and amidone as shown in table 2.
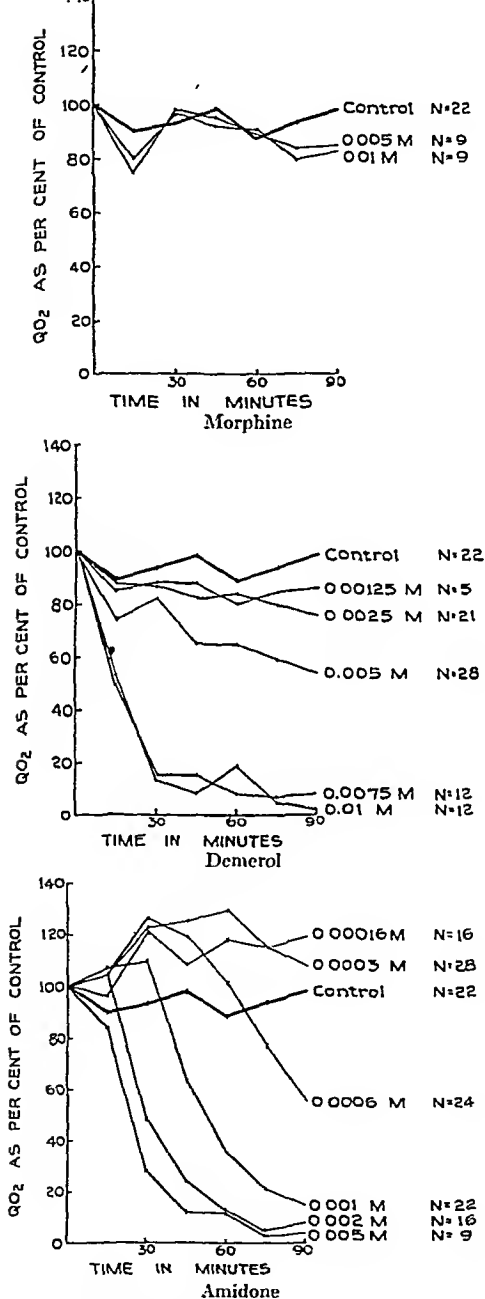
FIG. 1. THE EFFECT OF MORPHINE, DEMEROL AND AMIDONE ON THE OXYGEN UPTAKE OF BRAIN SLICES

N = Number of Vessels

QO_2 = ml of oxygen per gram of tissue per hr.

On the basis of present evidence it seems most probably that demerol and amidone inhibit brain tissue oxygen uptake by interrupting the "main line of biological oxidation" at the dehydrogenase level rather than at the flavoprotein level as is postulated for anesthetics and hypnotics.

COMMENT. In contrast to morphine, demerol and amidone exert marked effects on the oxidation of glucose by brain tissue *in vitro*. A difference in action is apparent in that only amidone is capable of stimulating oxygen con-

TABLE 1

The effect of amidone and demerol on anaerobic glycolysis of cerebral homogenate preparations

	VESSELS	$Q_G^{N_2}$*	PERCENT INHIBITION
Control	34	0.41	—
Amidone 0.002 M	18	0.17	59
Demerol 0.01 M	11	0.00	100

* $Q_G^{N_2}$ (wet weight) 60 min. after addition of drug. Ml. of CO_2 per gram of tissue per hour.

TABLE 2

The effect of amidone and demerol upon the oxidation of lactate and pyruvate by cerebral cortex slices

		CONTROLS	AMIDONE 0.001M	DEMEROL 0.01M
Sodium Lactate 0.02 M	Q_{O_2}	2.48	0.64	0.25
	percent inhibition		74	90
Sodium Pyruvate 0.02 M	Q_{O_2}	2.32	0.47	0.14
	percent inhibition		80	94

Each value represents the average wet weight Q_{O_2} of eight vessels 60 minutes after the addition of the drug.

sumption. If inhibitory action alone is considered, amidone is approximately ten times as potent as demerol. It is of interest that Scott and Chen (2) found the same ratio to hold in comparing the threshold analgetic dose for albino rats. However, the concentrations capable of inhibiting brain respiration *in vitro* were many times greater than might be expected after *in vivo* administration of the analgetic doses used (amidone 1 mg. per kg.; demerol 10 mg. per kg.).

No attempt will be made here to explain the stimulating action of amidone in low concentration on glucose oxidation. A similar action has been reported for the convulsant diphenyloxazolidinedione (16) in contrast to the purely inhibitory action reported for the central nervous system depressant propazone (di-n-propyl oxazolidinedione) (15). Comparison of structural formulas (figure 2) suggests that the stimulant effect is related to the presence of the benzene rings which are absent in propazone.

In spite of the varying effects of these drugs on oxygen uptake there is some similarity in their action upon the mechanisms of biological oxidation. Seevers and Shideman (13) found that morphine inhibits lactic, citric and glucose dehydrogenases, but not succinic and alcohol dehydrogenases. Our experiments show that demerol and amidone exert their inhibitory effects by action on dehydrogenases involved in glucose oxidation but do not interfere with the activity of succinic dehydrogenase.

Except for the findings on succinate oxidation there is no evidence that morphine, demerol and amidone exert their effects on oxidative processes in a manner similar to anesthetics and hypnotics. For this reason and because of the high concentrations required to produce *in vitro* effects it is felt that these analgetics do not conform to Quastel's theory of narcosis.
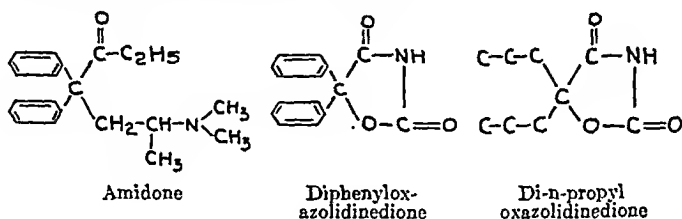
Amidone   Diphenylox-   Di-n-propyl
                       azolidinedione   oxazolidinedione
                       Fig. 2.

SUMMARY

The effects of demerol and amidone *in vitro* on the respiration and anaerobic glycolysis of brain tissue preparations have been studied by conventional manometric methods. Amidone is ten times as potent an inhibitor of glucose oxidation as demerol and stimulates oxygen uptake at low concentrations. The inhibitory action of these drugs is presumably due to an action on the dehydrogenases involved in glucose oxidation.

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A COMPARISON OF SOME ANTI-HISTAMINE SUBSTANCES

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A recent report by Dews and Graham (1) dealt with the properties of N-dimethylaminoethyl-N'-p-methoxybenzyl-aminopyridine which was described by Bovet and Walthert (2) under the name of "2786 R.P." and which is offered for clinical use as "Anthisan". This substance has remarkably high powers as an antagonist of many of the effects of histamine. It is not, however, specific: thus it does not prevent the relaxation of the smooth muscle of the uterus of the rat by histamine although it modifies the action of histamine in relaxing the smooth muscle of the coronary vessels of the cat and dog. It is a local anaesthetic, has a quinidine-like action on cardiac tissue and antagonises the action of histamine on the heart, blood vessels, bronchi and gut and on the uterus of some species.

Dimethylaminoethyl benzhydryl ether HCl or "Benadryl" was introduced by Loew, Kaiser and Moore (3) and has been investigated in some detail both experimentally (Loew *et al.* 4) and clinically (Feinberg 5). It has similar properties to "2786 R.P."

2-(N-phenyl-N-benzylaminomethyl)-imidazoline was introduced by Meier and Bucher (6) and is available for clinical use under the name of "Antistine". It is stated to be highly specific in its actions against the effect of histamine on the gut, blood vessels, bronchi, etc. and to be of value in conditions of allergy and skin diseases (Schindler 7; Brack 8).

All three compounds have been claimed to be of great value in minimising the effects of experimental anaphylactic shock.

Consideration of the structural formulae of these three potent antagonists of histamine, as shown below, demonstrates their similarity. All three are white crystalline solids: "Antistine" is less soluble than the other two.

1. "2786 R.P."
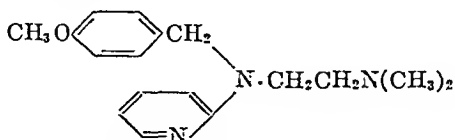
N-dimethylaminoethyl-N'-*p*-methoxybenzyl-aminopyridine

2. "Benadryl".
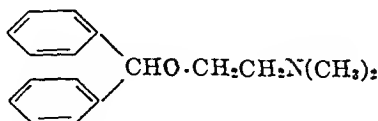
dimethylaminoethyl benzhydryl ether

3. "Antistine".
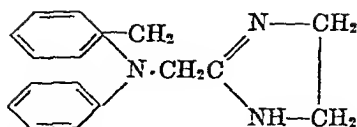
2(N-phenyl-N-benzylaminomethyl)-imidazoline

In view of the increasing use of these substances as therapeutic agents it was thought that an attempt at a quantitative comparison of their effectiveness against various activities of histamine might be of value and that an attempt to correlate this potency with other properties of these substances might be illuminating. Accordingly the potency of "2786 R.P.", "Benadryl" and "Antistine" against the action of histamine on the blood pressure of cats, the isolated intestine of guinea pigs and the bronchi of intact guinea pigs was investigated and the toxicity to white mice measured. The efficiency of these substances as local anaesthetics, antispasmodics and inhibitors of the histamine-induced flow of gastric juice was also investigated.

METHODS. 1. Cats were anaesthetised with ether and the blood pressure recorded from the left carotid artery. A standard dose of 2.5 µg. of histamine base (as acid phosphate) per kg. weight dissolved in 0 5 cc. saline was injected I.V. every two minutes and washed in quickly with 1.0 cc. of saline. After a few preliminary doses a very regular response was obtained of an acute fall in blood pressure of some 60 mm. Hg with a rapid recovery to the original level. The rate and depth of respiration was temporarily increased. When this response had been recorded 5–8 times the anti-histamine drug was given two minutes after the last injection of histamine in 0 5–1.0 cc. saline and allowed to act for two minutes, when histamine was given again. The animal was then rested for thirty minutes and the process repeated. In all five cats were used for each drug investigated, and three to four doses of each drug tested on each cat.

The mean fall in blood pressure caused by the five doses of histamine immediately previous to the injection of the anti-histamine drug was measured from the kymograph record and the difference between that mean figure and the figure for the fall in blood pressure caused by the same dose of histamine given after the anti-histamine drug gives sufficient data to plot the relation between log dose and per cent. response for each animal used. From each such curve a dose of anti-histamine drug such as would probably cause a 50% response (i e. reduction of the effect of the standard dose of histamine to half) can be obtained by interpolation, and the mean of the five determinations used as a potency figure for comparison with the other anti-histamine drugs tested

2. In investigating the anti-spasmodic activity of these compounds strips of terminal guinea pig ileum were used mounted in Tyrode solution at 38°C The effect of BaCl₂ (one in five thousand), acetyl choline (one in 75 million) and histamine base (one in 75 million) was determined as follows After the strip of gut was set up so that it showed little or no spontaneous movement and the concentration of spasm producing agent produced a submaximal contraction, this dose of the agent (BaCl₂ 5 mg., ACh 1 µg., H base 1 µg in a 75 cc. bath) was added every five minutes and allowed to act for one minute before washing out. After five equal successive responses to this agent had been obtained the antispasmodic drug under test was added in suitable dosage, allowed to act for two minutes, and the same standard concentration of Ba, histamine or choline ester added without washing out The effect of one antispasmodic compound in three concentrations was tested on the action of one spasm-producing agent in standard dosage on one piece of gut. Each agent was used twice on one guinea pig which thus provided six strips of gut. Each antispasmodic drug

maintained at 37.5°C and aerated by bubbling a mixture of 5% CO_2 and 95% O_2 through a side tube similar to that described by Harne (4). Washing is conducted by flooding upward from the bottom to avoid exposure to the air. Although ink-writing devices may be used, the studies described in this paper were made with a light, sensitive, 13-inch muscle lever yielding twelve-fold magnification. A short piece of aluminum wire coiled loosely about the end of the lever formed an almost frictionless writing point when used with lightly smoked paper. The kymograph was set to move at the rate of 0.1 cm. per minute.

The only tension required for the preparation is that needed to keep the chain vertical because the muscle fibers which stretch across the ends of the C-shaped ring of cartilage apparently are normally under a state of tension, for if they are sectioned, the ring springs open. It was found that satisfactory tension could be obtained as follows: The lever is first balanced by hanging small weights on the short arm. Then sufficient of these weights are removed to equal the weight of the chain. Finally the preparation is connected. For example, if the chain weighs 350 mgm., weights totaling 350 mgm. are removed. Under these conditions, the only tension used is that caused by the weight loss of the chain through the buoyancy of the bathing solution.

In studying the antispasmodic action of a drug, we observed first its effect on the normal or untreated trachea (see figure 1). Then its spasmolytic action was determined by its efficacy in relieving the spasms induced respectively by histamine phosphate, acetylcholine bromide and barium chloride (see figure 2).

RESULTS. In table 1 are summarized the results obtained on 72 guinea pig tracheas. The drug dilutions listed represent the dilutions of the antispasmodics which in typical experiments were found to produce a definite practical effect as defined in the legend of the table.

A Comparison of Bronchodilator and Spasmolytic Drugs on the Untreated Trachea. The three well-known bronchodilators, epinephrine, aminophylline and papaverine dilated the untreated trachea. In sharp contrast, the spasmolytics, atropine, Novatropine, Syntropan and Trasentin and the antihistamine drug, Benadryl, produced no relaxation (see figure 1). In fact, Benadryl in large doses caused contraction. Figure 1 also shows that epinephrine caused prompt relaxation but that after washing, its action was brief as shown by the rapid return to the original level. Aminophylline was much less potent from a dosage standpoint, as might be expected from clinical experience, but it was nevertheless promptly effective. Papaverine acted the most slowly, but its effect was quite prolonged, even after washing. It was found that graded responses could be obtained with graded doses of these three bronchodilator drugs. Work is in progress on the development of assays for bronchodilator drugs.

The Antagonism of the Bronchodilators toward Spasmogenic Drugs. All three bronchodilators counteracted the spasmogenic drugs, histamine, acetylcholine and barium chloride, probably by virtue of their ability to relax tracheal muscle rather than by any specific drug antagonism. That is, the effect obtained may be considered the resultant of two drugs acting independently but in opposite directions. As figure 2 shows, epinephrine was rapid but evanescent in action, even without washing. Aminophylline was also rapid in onset, but its effects were more prolonged and papaverine was both slow in onset and of long duration. Table 1 shows the very wide difference in the potencies of these three bronchodilators.

Atropine. This drug produced no visible effect of its own on the tracheal muscle. In sufficiently high concentrations (1:50,000), it inhibited the action

TABLE 1

Maximal dilutions of the antispasmodics that under the conditions described in this paper produced (I), a dilatation of the normal or untreated trachea sufficient to cause approximately a 1 cm. fall in the tracing, and (II), a definite relief (75 to 100 per cent) of the constrictions induced by histamine, acetylcholine and barium chloride

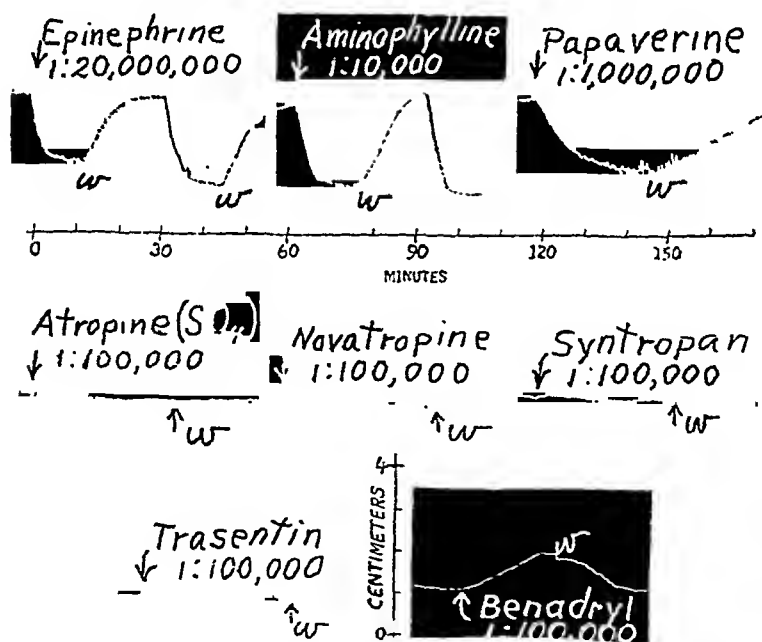| ANTISPASMODIC	I	II		
	Normal Muscle	Histamine Phosphate 1:500,000	Acetylcholine Bromide 1:1,000,000	Barium Chloride 1:5000
Epinephrine	1:100,000,000	1:20,000,000	1:20,000,000	1:80,000,000
Aminophylline	1:200,000	1:25,000	1:5000	1:20,000
Papaverine	1:2,000,000	1:500,000	1:400,000	1:500,000
Atropine Sulfate	None	1:50,000	1:50,000,000	None
Novatropine	None	None	1:20,000,000	None
Syntropan	None	None	1:200,000	None
Trasentin	None	None	1:125,000	None
Benadryl	None	1:15,000,000	1:250,000	None

FIG. 1. THE EFFECT OF THE INDICATED CONCENTRATIONS OF THE ANTISPASMODICS ON THE NORMAL OR UNTREATED TRACHEAL CHAIN. (W) INDICATES WASHING

of histamine but it was found to be a thousand times more potent in counteracting acetylcholine (see table 1). Atropine failed to alleviate the spasms of

pavatrine and demerol are much less specific in their antispasmodic activity. While all three anti-histamine compounds are potent in inhibiting histamine, "2786 R.P." is the most active against histamine and relatively poor against Ba and ACh "Benadryl" though almost as active as "2786 R.P." against histamine is remarkably active also against Ba and ACh in which respects it resembles demerol in potency. "Antistine" is a less active antispasmodic than the other two though it is more potent that "2786 R.P." in inhibiting cholinergic activity. It is apparent that Ba H and ACh have different "receptor points" in the cell. A compound which is highly specific in blocking one type of receptor is unlikely to be equally specific in attacking another type of receptor.

The Anti-Histamine Potency (1) as assayed on spasm of the guinea pig bronchus. "2786 R.P." proved to be the most potent agent in diminishing the

TABLE 2

Relationship of dosage of antihistamine compound injected s.c. to the per cent increase in the mean time required for a spray of two per cent histamine base to cause asphyxia in a group of guinea pigs; and an approximate estimate of their relative potencies in this respect

COMPOUND	DOSAGE	% RESPONSE	RELATIVE POTENCY ("ANTISTINE" EQUALS ONE)
	mg./kg.		
"2786 R.P."	0.025	114	
	0.10	128	
	0.20	140	4
"Benadryl"	0.025	52	
	0.10	62	
	0.20	82	2
"Antistine"	0.025	20	
	0.10	33	
	0.20	45	1

effect of histamine spray on the bronchi of a group of guinea pigs as measured by the increase in the mean time taken to cause asphyxia in the animals when subjected to a spray of 2% histamine base. The essential results are shown in table 2 and figure 1 from which it can be seen that "2786 R.P." is approximately four times as potent as "Antistine" in this respect. All three compounds have some protective effect and as the slope of the curves is not steep a large degree of protection can be obtained with a relatively small dose.

(2) As assayed on the blood pressure of the cat "2786 R.P." proved to be the most potent of the three compounds in reducing the amount of the fall in blood pressure of the cat anaesthetised with ether which follows upon injection of histamine. The degree and duration of the fall was very constant as is seen in figure 2 and the doses of anti-histamine compounds had lost their effect on the action of histamine on the blood pressure after thirty minutes. The degree of scatter of the results for any one compound tested on five cats was small and the

slope of the curves for all three compounds parallel (see fig. 3).   Table 3 shows the mean doses of these compounds required to reduce the mean effect of 2.5 μg histamine base per kg. body weight in groups of five cats anaesthetised with
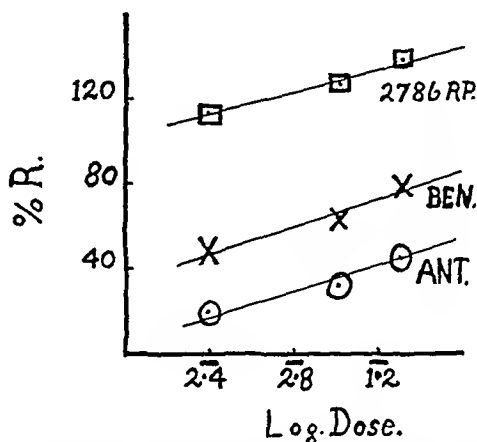
FIG 1. Relation of the log dose of antihistamine agent (abscissa) to per cent response (ordinate) of degree of protection offered to guinea pigs exposed to two per cent histamine base in a fine spray.   All three agents are effective but "2786 R P" is the most potent.
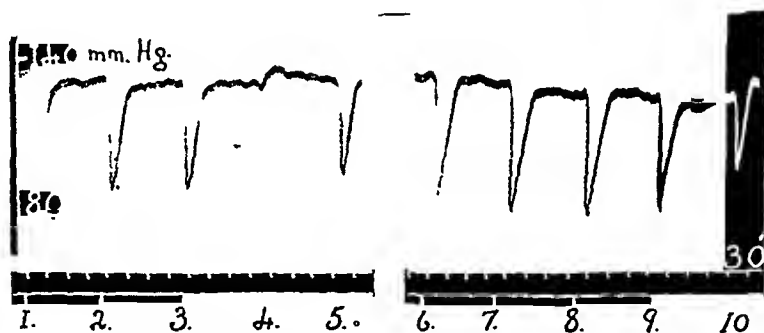
FIG 2 Cat, ♂, 3 4 kg   Ether anaesthesia   From above, carotid B Pr , time in 30 secs , injection signal   Histamine base 2 5 μg /kg weight i v. every two minutes at 1 2.3 5. and 6 7 8 10   At 4 "Antistine" 0 25 mg /kg and at 9 "Antistine" 1 0 mg /kg   Between 5 and 6 pause of 30 mins   Note the regular response to histamine (about 60 mm Hg fall in B Pr.); the rise in B Pr from a small dose and the fall in B Pr from a large dose of antihistamine compound, the proportional inhibition of the response to histamine, and the recovery from this effect within 30 mins

ether and gives an approximate estimate of their relative strengths   It will be seen that "2786 R.P" is nine times as potent as, and "Benadryl" rather less powerful than "Antistine."

   Local Anaesthesia.   The strength of these compounds as local anaesthetics

was estimated by the method of Bulbring and Wajda (9) using intra-cutaneous wheals in groups of guinea pigs. Procaine in solutions of 0.1%, 0.25% and 1% was compared with "2786 R.P.", "Benadryl" and "Antistine' in concentrations of 0.05%, 0.1% and 0.25%. If the potency of procaine is considered to be unity, "2786 R.P." is 3.3 times as strong, "Benadryl" 2.5 times as strong and "Antistine 1.5 times as strong. This agrees with the previous test of "2786 R.P."
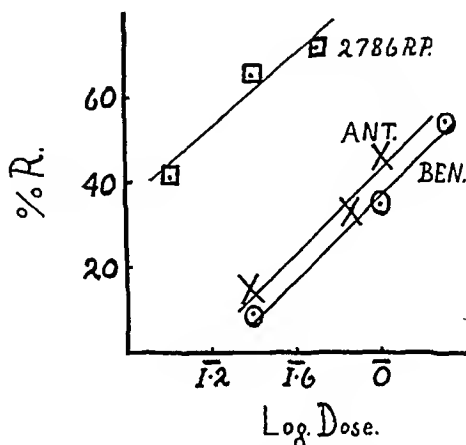
Fig. 3. Relation of the log. dose of antihistamine agent (abscissa) to the per cent response (ordinate) of the degree of inhibition of the mean fall in carotid blood pressure of groups of etherised cats due to i.v. injection of histamine base 2.5 µg /kg. "2786 R.P." is the most active.

TABLE 3

Quantities of antihistamine compounds required to reduce by one half the mean depression of the carotid blood pressure of groups of cats onaesthetised with ether and injected i.v. at two minute intervals with 2 5 µg histomine bose per kg. bodyweight

COMPOUND	QUANTITY REQUIRED TO GIVE A 50% RESPONSE	RELATIVE POTENCIES ("ANTI STINE" EQUALS ONE)
	mg /kg	
"2786 R.P."	0.14	9.3
"Benadryl"	1.7	0.76
"Antistine"	1 3	1.0

carried out at another laboratory by Dews and Graham (1) and explains the findings of Bourquin (10) that this substance could be used to replace cocaine in the examination of irritated and inflamed eyes.

Gastric Juice. In view of the fact that injected histamine produces a flow of gastric juice in the human stomach the report of Loew et al. (4) that "Benadryl" had an effect on the flow of gastric juice in dogs after histamine opened up possibilities of a new therapy of human cases of acid dyspepsia.

The results obtained from groups of 12 young guinea pigs treated as described showed that in all animals isolation of the stomach lumen produced a state of ileus with a copious fluid content which contained no free HCl and little combined acid. The difference noted after histamine was the appearance of free acid in two thirds of the animals and the great increase in the combined acid in all. None of the anti-histamine compounds in a dosage of 4 mg./kg. s.c. abolished this free acid or reduced the combined acid in the gastric contents but "2786 R.P." was the most active in reducing the free HCl found after histamine from a mean figure of 0.4% N/1 HCl in the total control group to a mean figure of 0 05% N/1 HCl in the treated group, present in less than one tenth of the cases. It appears that "2786 R.P." is more active than the other two but that all reduce the free acid somewhat. None of these substances in a concentration of 1 in 150,000 regularly inhibits the spontaneous movement of isolated human gastric muscle.

DISCUSSION. Loew, Kaiser and Moore (3) found when investigating the anti-histamine activity of a series of benzhydryl ethers that compounds with a chain length of 2 carbon atoms in the ethylamine side-chain gave optimal activity and that potency was lessened if the chain was branched. Tertiary amines were most active if the amino group was simple. This is confirmed by the greater anti-histamine potency of "Benadryl" and "2786 R.P." than "Antistine" in which the N. is complex. Loew et al. further found in their series of benzhydryl ethers that substitution on the benzene rings of the benzhydryl group reduced the potency of such compounds. A comparison of pyribenzamine (Mayer et al. 11) with "2786 R.P." from which it differs only in the absence of a methoxy group on the ring would be of interest. Both are very potent anti-histamine agents.

The difference between the aralkyl groups of "Antistine" and "2786 R.P." is much less than the difference between these groups and the similar group in "Benadryl". Since "Benadryl" and 2786 R.P." are roughly equal in anti-histamine potency despite the difference in their aralkyl groups and the linkages, the side chain must be the main key to anti-histamine activity. An ether ("Benadryl") or a N- linkage ("2786 R.P.") between the chain and the cyclic groups appears to be equally effective. N-methyl end groups on the chain appear to be more potent than the imidazole structure. Winder et al. (12) found tertiary amines of benzhydryl ethers to be convulsant but quaternary salts to be depressant and suggest for the latter a possible curariform action (c.f. Ing et al. 13); the ataxic-depressant effect of "Antistine" suggests that a quaternary salt is not necessary for this property to appear which must be related to the imidazole substitution at the end of the chain.

The fact that the most potent histaminolytic compound ("2786 R.P.") is the most active local anaesthetic is of interest in relation to the theory that a histamine-like substance plays some part in the activation of pain receptors in the skin. The action of "2786 R.P." in inhibiting the amount of free HCl in the stomach after histamine injections indicates that this substance might be worthy of trial in acid dyspepsia, on the basis of a suggestion that histamine plays some part in the production of gastric juice. None of these compounds is entirely

specific in its action against histamine as all three fail to modify the inhibition of isolated rat uterus which results from added histamine. Increasing the concentration of these drugs has an oxytoxic effect. "Benadryl" is the most toxic when given intraperitoneally to mice but the difference between the three is only moderate. The fact that "2786 R.P." and "Benadryl" are stimulant to the C.N.S. and "Antistine" is depressant is important. The drug of choice for clinical use in allergic states would appear to be "2786 R.P." or "Benadryl"; the former is alleged to have less unpleasant side effects in the way of drowsiness and dizziness.

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AMEBACIDAL AND PHARMACOLOGIC ACTIVITIES OF CARBARSONE OXIDE (P-CARBAMIDOPHENYLARSENOUS OXIDE) AND ITS DITHIOCARBOXYMETHYL AND DITHIOCARBOXYPHENYL DERIVATIVES*

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Ehrlich suggested in 1909 (1) that the toxic action of arsenicals might reside in their affinity for thiol groups. This hypothesis was explored by Voegtlin, et al., in 1923 (2) who believed that arsenoxides react with reduced glutathione, either in tissues of the host or of the invading parasite. Cohen, King and Strangeways (3) in 1931 produced a series of dithioarsenites, in the belief that there was justification for the opinion that the lethal action of arsenic on tissues is a chemical one and might well relate to thiol groups.

Among the aromatic thioarsenites described by Cohen, et al. in 1932 (4) and Strangeways in 1935 (5), there were two compounds active against *Trypanosoma rhodesiense* and *Trypanosoma gambiense* infections in mice. Subsequent studies by Murgatroyd (6) in man, however, revealed that the toxic effects produced by these agents precluded their clinical application.

Because the hypothesis of Cohen, et al., appeared to be attractive, chemical and biologic studies have been conducted by other workers. It was believed that the thioarsenites slowly liberated an active arsenoxide. The greater therapeutic usefulness of the thioarsenites might well be dependent on slow formation of the arsenoxide which implies sustained exposure of the parasite to effective amounts. Partial hydrolysis has been demonstrated within 30 minutes and this continued over an extended period. Conceivably, the tissues of the host are capable of detoxifying the arsenical more rapidly than are those of the parasite. While this reasoning has not justified the introduction of antitreponemal or trypanocidal agents, we believe evidence has been obtained to support the introduction of potentially useful amebacides.

AMEBACIDAL ACTIVITY. Methods utilized to demonstrate amebacidal activity *in vitro* and *in vivo* have been reported previously (7). These included exposure over 48 hours at 37°C. of a culture of trophozoites of *E. histolytica* plus a single bacterium, organism "t," to the drug under test in a suitable medium. Cultures in egg slope and liquid liver medium have been made. The

concentration of carbarsone (p-carbamidophenyl arsonic acid) required to exert amebacidal activity was 1:2,000–1:4,000. For emetine hydrochloride, 1:20,000 dilution was amebacidal. Its dithio derivatives[1] were within the range of activity of emetine, and for the oxide, there was appreciably greater activity in the egg slope medium.

Against natural infections of *E. histolytica* in macaques, oral or parenteral application of each of these agents was tried. Toxicity studies in mice, rats and rabbits (later given in detail) were used to determine the approximate range of dosage for monkeys. During this phase of the study, the clinical pathologic effects of the arsenoxide and its dithio derivatives were observed (see later

TABLE 1

Amounts of test agents required to clear macaques of natural amebic infection

COMPOUND	ORAL DOSE IN mgm./kgm. (TOTAL GIVEN / NO. OF DAYS)	
Carbarsone (p-carbamidophenyl arsonic acid)	30 mgm./kgm. / 23 days	Partially effective in 3/8
Carbarsone oxide (p-carbamidophenyl arsenous oxide)	11–40 mgm./kgm. / 5 to 30 days	Effective in 3/5
Phenyl urea p-di-(carboxymethyl) thioarsenite (C. C. #914)	20–50 mgm./kgm. / 8 to 30 days	Effective in 5/5
p-Carbamidophenyl-di(1'-carboxyphenyl-2') thioarsenite (C. C. #1037)	20–50 mgm./kgm. / 8 to 30 days	Effective in 5/5
Emetine hydrochloride	3–10 mgm./kgm. / 5 to 8 days	Partially effective within toxic range in 2/2

* All agents were given orally, period of observation was three months.

section on tolerance tests). Table 1 summarizes these data on *in vivo* activity levels.

On the basis of these observations it may be noted that carbarsone oxide was more effective than its pentavalent analog, carbarsone. Likewise, the two dithio derivatives proved equally active, and were successfully employed in the dose range chosen. The reference standard, emetine hydrochloride, could not be given in effective amounts without producing nausea, vomiting and evidence of cardiac damage.

[1] Prepared by the Lilly Research Laboratories. The compounds were submitted by the Chemotherapy Center, Office of Scientific Research and Development, as C C. 914 and 1037, and have been termed thioarsenites. C C 914 has also been named p-[bis-(carboxymethyl-mercapto)arsino]phenylurea and C C 1037 as p-[bis-(o-carboxyphenylmercapto)arsino] phenylurea

PHYSICAL-CHEMICAL CHARACTERISTICS. The physical-chemical characteristics of carbarsone oxide and its dithio derivatives were determined. The data summarized in table 2 are on lots used throughout the study.

TOXICITY TESTS. In an attempt to determine the toxicity of these arsenicals for host tissues, rabbit leukocytes were exposed to varying concentrations at 37°C. Emetine hydrochloride, as a reference standard, did not alter motility of white cells during 30 minutes exposure at 1:1,000 dilution. Carbarsone oxide, at this dilution, did not immobilize the cells but pseudopodia appeared abnormal. For the two dithio derivatives, 1:500 concentrations did not affect motility during the 60-minute test period.

Acute toxicity levels for mice and rats were determined by various routes. Table 3 summarizes these data. Toxic or tolerated levels in other species are

TABLE 2

Physico-chemical characteristics of carbarsone oxide and its dithio derivatives

COMPOUND	M. P.	M. W.	SOL IN WATER	SOL. IN IN. HCl	SOL IN IN. NaOH	SOL IN C₂H₅OH	% ARSENIC FOUND	% SULFUR FOUND
Carbarsone oxide (p-carbamidophenylarsenoxide)	Decomposes at ca 262°C.	226	very slight	Insoluble	1–2%	Insoluble	32 1–33 8	none
Phenyl urea p-di-(carboxymethyl) thioarsenite (C C. 914)	83–84°C	392	very slight	Insoluble	33%	5%	20 74*	15 93*
p-Carbamidophenyl-di-(1'-carboxyphenyl-2') thioarsenite (C C 1037)	158°—160°C.	516	very slight	Insoluble	20%	5%	14 75*	11.42*

* Supplied by Dr. Ewald Rohrmann, Lilly Research Laboratories

included also, although an insufficient number of animals was used to determine exact LD₅₀s, except in mice and rats. For the toxicity tests in mice, 537 animals were used; for the toxicity tests in rats, 575.

Rabbits were given carbarsone oxide in daily doses of 2.2, 5.3 and 13.3 mgm./kgm., in enteric coated tablets. Changes in appetite, weight, hepatic function as measured by the bromsulfalein test, and renal function, as measured by a phenolsulfonphthalein test,[2] were observed. The two smaller doses did not
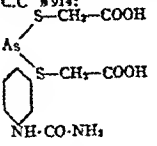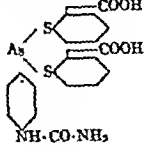
[2] Test of kidney function in the rabbit (F. P. Ludueña) performed by Dr. B. E. Abreu:
1. Give the animal 50 ml. of distilled water per kg , intragastrically.
2 30 minutes later catheterize and empty the bladder
3. Leave catheter in place for 1 25 hours Collect and save this urine for color control.
4. Inject phenolsulfonphthalein P S P , 0 5 ml of 0 03% /Kg. (0 15 mgm /Kg) I.V.
5 Collect urine for 2 5 hours
6 Into 3-100 ml volumetric flasks.
   (a) put control urine in #1 flask and dilute to 100 ml. after adding 2–3 ml. of 10% NaOH.

TABLE 3

Toxic and tolerated doses of carbarsone oxide and its dithio derivatives
(C. C., #914 and #1037) given in single doses (expressed as mgm./kgm.).

SPECIES AND ROUTE USED	CARBARSONE OXIDE $As=O$ ⬡ $NH \cdot CO \ NH_2$	C.C #914: $S-CH_2-COOH$ As ⬡ $S-CH_2-COOH$ ⬡ $NH \cdot CO \cdot NH_2$	C.C. #1037: S⬡$COOH$ As S⬡$COOH$ ⬡ $NH \cdot CO \cdot NH_2$
Mice, I.V., LD_{50}	41.3 ± 1.4*	42.6 ± 1.9*	120 ± 6.2
Mice, I.P., LD_{50} . . .	59 ± 6.7	100 ± 9.0	265 ± 12.2
Rats, I.V., LD_{50} . . .	17	29	70
Rats, I.P., LD_{50}	55 ± 2.1	75 ± 4.7	76 ± 4.1
Rats, I.G., LD_{50}	510 ± 40	1000 ± 39	1220 ± 54
Rabbits, I.V., (lethal range)	20 — 40	100	100 — 300
Rabbits, I.G., (tolerated dose)	75+	300+	200 — 300
Monkeys, oral (tolerated daily dose)	40±	50±	50±
Man, oral, (tolerated daily dose) . .	90 mgm. (total)	100 mgm. (total)	100 mgm. (total)

* Observations of Dr. K. K. Chen, Lilly Research Laboratories.
I.P. = Intraperitoneal; I.V. = Intravenous; I.G. = Intragastric.

(b) put 2.5-hour sample in #2 flask, add 2–3 ml. of 10% NaOH and dilute to 100 ml.
(c) put volume of P.S.P. solution injected into rabbit into flask #3; add 2–3 ml. of 10% NaOH and dilute to 100 ml.
7. Calculation:
(a) Read urine from control flask in photoelectric colorimeter.
(b) Read urine from 2.5 hour-sample flask in photoelectric colorimeter.
(c) Read standard solution in photoelectric colorimeter.
Subtract the reading for the control flask from that of the 2.5 hour sample. Divide the reading from the 2.5 hour sample by that of the standard solution and multiply by 100. This value gives per cent recovery of P.S.P. in urine.

cause any alterations in weight, function or appearance of the animals over 34 days. After 22 days, however, the rabbit given 13.3 mgm./kgm. daily, died. These findings were included in an earlier preliminary report (8).

PATHOLOGIC STUDIES. Tissues from rabbits given single intragastric lethal doses of carbarsone oxide revealed no significant damage except perisplenitis and focal necrosis of the lungs. The gastric mucosa was hyperemic and had petechial hemorrhages. In rats, gastric irritation and hemorrhage followed single intragastric administration of carbarsone oxide, but there was no evidence of damage to tissue elsewhere in the gastro-enteric tract. The liver was moderately emigested.

The single intraperitoneal administration of carborsone oxide to rats and mice resulted in an acute toxic reaction characterized by the formation of an exudate in the peritoneal cavity Surviving animals developed an acute inflammatory reaction with the formation of fibrous adhesions The pleural transudate which formed was apparently resorbed without permanent pathologic changes in the tissues.

In the monkey, parenteral application of toxic amounts of carbarsone oxide resulted in massive acute liver degeneration and early hemorrhagic pulmonory edema. A low-grade meningitis of the compound granular cell type reaction was observed in one animal.[3]

The gastric mucosa of rats sacrificed on termination of the short-term chronic iotragastric toxicity test was normal. Albuminoid changes in the secretory cells of the kidneys were the only abnormalities noted and were present in animals sacrificed nine days after the last dose given

C C 914 given as a single intraperitoneal injection of a toxic amount to rats produced only a general, serous exudate, and an acute toxic reaction in the liver, lungs and kidneys. Necrosis of the renal tubules was observed.

Oral administration of single, toxic doses of C.C. 914 to rats resulted in no significant changes in the gastric mucosa. There was slight toxic degeneration of the renal tubules. The lungs were congested and atelectatic The mucosa of the cecum was normal; the villi of the duodenum were edematous.

C.C. 1037 given as intraperitoneal injections of single toxic doses in mice produced a serous exudate in the lungs, as well as hyaline degeneration of the renal tubules and congestion of the glomeruli In rats, the same route of administration of the compound caused no evidence of central nervous system reaction; the kidneys, hearts, lungs and spleens, as well as the duodena, were normal Slight toxic degeneration of the secretory tubules of the kidneys was observed Survivors showed no pathologic changes.

After oral application of toxic doses of C.C 1037 to rats, the gastric mucosae appeared unchanged. The livers revealed fatty degeneration There was slight toxic necrosis of the renal tubules, and slight pulmonary edema The spleens appeared edematous.

Each of the dithio derivatives, C.C 914 and 1037, revealed essentially the same character of pathologic effect in sacrificed animals on the short-term chronic toxicity test. Damage to the convoluted tubules was observed. These were described as cloudy, granular changes with vacuolization of the cytoplasm. The nuclear structure was normal. This was described by the pothologist as albuminoid degeneration and as definitely reversible at this stage. In animals on the short-term chronic toxicity test, some evidence of damage persisted nine days after cessation of therapy. No pathologic changes were observed in the liver, spleen, pancreas, cerebrum and cerebellum or in the cardiac muscle.

ARSENIC DISTRIBUTION STUDIES AND CHRONIC TOLERANCE TESTS. In order to learn of the distribution of arsenic after application of carbarsone oxide and its two dithio derivatives, modifications of the method of Cassil and Wichmann (9) were used. The modifications were concerned with the wet acid digestion,

[3] As observed by Drs. Warren Bostick and E Lowenhaupt

as suggested by James W. Hansen, and again modified by one of the authors (P.P.T.S.). These were: (1) perchloric acid was omitted in the final digestion because of its explosive character and the possible loss of arsenic as $AsCl_3$ and $AsCl_5$ by volatilization; (2) the last trace of nitric acid was removed from the digested sample by addition of hydrogen peroxide; (3) for greater accuracy, the arsenic content of the aliquot in the distilling flask was adjusted to from 10 to 25 micrograms. The values reported in the accompanying tables were found to be accurate within 2 per cent.

When dealing with small animals, about ten grams of tissue were placed in a Kjeldahl flask and heated with 1 ml. of concentrated nitric acid. Twenty-five ml. of concentrated sulfuric acid and about 4 grams of potassium sulfate were added and the mixture was boiled until a black concentrated solution was obtained (in about 45 minutes). Concentrated nitric acid was then added, first dropwise and then in volume, until a pale yellow or water-clear solution was obtained (usually about 10 ml. were required). The mixture was boiled until dense white clouds of sulfur trioxide appeared in the flask. Fifty ml. of water were then added to the cooled solution, and the material was again boiled until heavy white fumes of sulfur trioxide appeared.

In later studies with monkeys on the dithio derivatives, C.C. 914 and 1037, greater accuracy was obtained. The entire organ used for analysis (except muscle, urine, blood and skin), after weighing, was placed in a large Erlenmeyer flask and covered with arsenic-free concentrated nitric acid. After standing for 24 hours at room temperature, 25 ml. of concentrated sulfuric acid were added with shaking. The contents were warmed gently on the water bath (about 60°C.) for 48 hours at which time the tissue was completely disintegrated. The contents were transferred to a Kjeldahl flask and the Erlenmeyer flask was carefully washed five times with small amounts of concentrated nitric acid. The total digested solution was then boiled over a small flame until the volume was about 30 ml. In a majority of cases, a clear solution was obtained. With liver, omentum or brain tissue, which contain much lipid, it was necessary to add more nitric acid and to prolong the digestion. When dense white clouds of sulfur trioxide appeared and the solution became clear and colorless, 50 ml. of distilled water were added and the solution was again boiled until heavy white fumes of sulfur trioxide reappeared. The last trace of nitric acid was driven off by the addition of a few ml. of superoxol and by boiling. After cooling, the solution was transferred to a 100 ml.-volumetric flask and made up to volume with distilled water. Ten ml. of this sample solution were removed for analysis. If the arsenic content was extraordinarily high, the sample was further diluted ten times and a suitable aliquot taken for analysis. With practice, the accuracy of the method could be brought to within 2 per cent.

Table 4 summarizes the data in rabbits which received carbarsone oxide, in single intravenous doses of 20 and 40 mgm. per kgm. and at a dose of 75 mgm. per kgm., given intragastrically. The extent of absorption after intragastric application is reflected in the surprisingly high levels of arsenic obtained in the bile, as well as in the urine, kidneys, cecum, liver, and bone marrow.

The fate of arsenic in the rabbit after intravenous injection in about one-tenth the amount, as m-amino-p-hydroxyphenylarsenoxide, resulted in comparable distribution, according to Chance, Crawford and Levvy (10). The single exception was the urine level which was considerably higher in their studies.

Regardless of whether the arsenicals were given intragastrically or intravenously, fairly high levels were established in the bile and bowel wall and contents where they would be required to exert amebacidal action. Further,

carbarsone oxide persisted in the tissues as long as 9 days after cessation of the short-term chronic toxicity test in rats.

Chronic tolerance tests in rats and monkeys were conducted, following the short-term test described by Carl C. Smith (11). Tables 5 and 6 summarize the growth rates and arsenic levels during and after completion of the test, and the comparative arsenic levels obtained with carbarsone oxide and its dithio derivatives in rats. In animals treated with carbarsone oxide, gains in weight

TABLE 4

Arsenic distribution in rabbits after single doses of carbarsone oxide
(Expressed as As_2O_3 in micrograms per gram of wet tissue)*

TISSUE OR SUBSTANCE TESTED	20 mgm /kgm GIVEN I V †	40 mgm /kgm GIVEN I V †	75 mgm /kgm INTRAGASTRICALLY‡
Bile			198
Cecal contents	68 6		125 4
Colon contents	17 2	47.5	92 4
Stomach contents		0.4	72 6
Urine	27	1 3	35 3
Kidneys	33	66	3 5
Spleen		23 8	1 1
Cecum	22 4	68 6	4 5
Colon	13 1	44 9	0 9
Liver	9 2	105 6	2 9
Small bowel	8 8	10 6	2 9
Stomach		11 8	1 1
Blood .		1 5	6 6
Duodenal contents	6 7		
Brain and cord	1 1		1 2
Lungs	2 1	6.5	0 4
Heart	1.1	4 1	0
Bone marrow			2 9
Muscle	1 3	1.3	0 1

* Results of Victor Bond, using the method of Cassill and Wichmann, J of Offic. Agric Chem , *22* 436, 1939

† Animals sacrificed 24 hours after therapy

‡ Died within 18 hours

were slightly less than in the controls. During the ten days subsequently, weights continued to increase within the range of the controls. For the dithio derivatives, the gains noted for the treatment period were not as great, but during the subsequent 10 days, resumption of growth rate approached that of the controls, especially in the group given C.C. 1037.

Arsenic distribution in rats, after intragastric administration of carbarsone oxide, was highest in the urine, blood, feces, lungs, heart and kidneys; the liver, colon and brain contained smaller amounts. The dithio derivatives, given in a single intravenous dose, established lower levels but in the approximate order noted for carbarsone oxide.

Carbarsone oxide, given orally to macaques in amounts of 11, 17 and 27 mgm./

kgm., respectively, for 30 times during a period of two months, likewise caused no signs of toxicity or alterations of the laboratory tests listed above. Each animal gained weight, an average of about 10 per cent during two months. Grossly, when sacrificed animals were autopsied, there were no significant changes noted that could be attributed to either the disease or the drug. Microscopic examination of tissues revealed fatty infiltration of the liver and degenerative changes in the renal tubules of one monkey which received the largest amount of the arsenoxide.

TABLE 5

Growth of rats given carbarsone oxide, C.C. 914 or 1037 during short-term chronic toxicity test

COMPOUND (GIVEN DAILY FOR 11 TIMES)	AVERAGE WEIGHT CHANGE IN GRAMS ON:	
	day following last dose	tenth day following last dose
CARBARSONE OXIDE		
1/16 LD$_{50}$	+19	+45
2/16 LD$_{50}$	+27	+33
3/16 LD$_{50}$	+26	+43
C.C. 914		
1/16 LD$_{50}$	+20	
2/16 LD$_{50}$	+22.5	
3/16 LD$_{50}$	+14.5	+29.5
C.C. 1037		
1/16 LD$_{50}$	+28	
2/16 LD$_{50}$	+31.5	
3/16 LD$_{50}$	+26.5	+48.5
Controls (no drug).	+36.5†	+50†

* Described by Carl C. Smith, Malaria Report #316, Feb. 12, 1945, for the Board for the Coordination of Malarial Studies.

† Compare with animals given C.C. 914 and 1037.

Arsenic distribution, expressed as As_2O_3 in micrograms per gram of wet tissue, after 5 daily intramuscular injections of 12 mgm. of carbarsone oxide per kgm. resulted in the following levels in monkey tissues: Liver, 45; feces, 30; kidney, 12; stomach, 4; blood, 3; lungs, 2; heart, 2; small bowel, 2; colon, 2; muscle, 0.2; and brain, 0.1.

Six monkeys (*Macaques rhesus*) were given orally on alternate days for 30 times, over a two-month period, 20, 30 and 50 mgm., respectively, of the dithio derivatives, C.C. 914 and 1937, per kgm. Each animal gained weight during therapy. No evidence of nausea, vomiting, anorexia or other disorder was observed. Red cells, hemoglobin, differential white count, bromsulfalein hepatic function test, blood urea, and the electrocardiogram were not altered during the treatment period. Animals sacrificed at conclusion of therapy revealed no

evidence of amebic infection. Other gross and microscopic changes noted were fatty infiltration of the liver in one monkey given 50 mgm. of C.C. 1037 per kgm., daily, and in two of the animals given larger doses of C.C. 914. In one macaque given daily doses of 30 mgm. of C.C. 914 per kgm., albuminoid degeneration of the convoluted tubules of the kidneys was noted. No other gross or microscopic evidences of amebic or drug effects were observed. The distribution of arsenic in these animals is summarized in table 7.

DISCUSSION. The thioarsenites included in this study appear to exert much of the anti-amebic activity of carbarsone oxide both *in vitro* and *in vivo*; at the same time they have exhibited relatively less toxicity for various mammalian species. Certainly the dithiocarboxyphenyl derivative (C.C. 1037) was significantly less toxic for mice, rats (I.V. and I.G.) and rabbits. In monkeys,

TABLE 6

Arsenic distribution in rats* after carbarsone oxide, 30 mgm /kgm., given intragastrically daily for 11 times; and after C C 914 or 1037, given in a single dose of 100 mgm /lgm, I V †

TISSUE OR SUBSTANCE TESTED‡	CARBARSONE OXIDE	C C 914	C C. 1037
Urine	250	119 1	130.4
Blood	145	12 8	15 8
Feces	80 5	17	3 1
Lungs	66	10	10 7
Heart	41	21 4	3
Kidneys	28	97 9	39 6
Liver	13	38 7	29 5
Colon	5.3	4 7	1 1
Brain	0.5	2 7	0
Pleural fluid		17 2	3 4

* Pooled tissues from 4 rats used for each drug

† Animals sacrificed or died within 2 4 hours of last dose

‡ From ten animals in each group

both dithio derivatives could be used beyond the necessary therapeutic range (±25 mgm /kg, orally) without impairment of any of the functions tested.

Distribution studies in three species revealed surprisingly high levels of arsenic, after carbarsone oxide, given either parenterally or intragastrically, in the bile, cecal, colon and stomach contents, urine, kidneys, cecum, colon, liver, small bowel and stomach. As with other arsenicals, when carbarsone oxide and its dithio derivatives were given in single doses intravenously, they were bound in the liver and kidneys. However, after oral application, smaller amounts were detected in these vital tissues and less evidence of damage was observed.

On termination of the short-term chronic toxicity test in rats, relatively high tissue levels were found as long as 9 days after cessation of therapy. Chance, et al. (10) have reported appreciable arsenic levels one week after injection of phenylarsenoxide in rabbits. Adequate absorption occurred after intragastric use; tolerated doses developed urine levels of arsenic comparable to those follow-

ing intravenous application.   The finding of high levels of arsenic in the bile, with each of the trivalent arsenicals, would favor their possible application in chronic disease of the gastroenteric tract; e.g., amebiasis.   The low levels of arsenic detected in the brain, together with absence of signs of disturbance of the central nervous system and of pathologic changes, again favor their use.

The pathologic changes produced by single doses of carbarsone oxide were far more devastating in all species studied (mice, rats, rabbits and monkeys) than was the case with either of the dithio derivatives.   Tissue reaction was noted

TABLE 7

Arsenic distribution in m nkeys which received orally, 20, 30 and 50 mgm./kgm. of C.C. 914 or 1037, in 30 doses over two months*

TISSUE OR SUBSTANCE TESTED	C.C. 914			C.C. 1037		
	20	30	50	20	30	50
	mgm./Kg.			*mgm./Kg.*		
Feces (in colon)	20.4	25.3	22.8	6.45	58.8	76.8
Contents of small intestine	8.9	2.2	5.8	1.0	1.2	2 9
Contents of stomach	1.8	0.75	0.6	1.7	1 4	2.2
Bile	10 0	12.7	108 0	13.9	5.3	0
Kidney	1 5	1.1	0	1.0	0	0
Urine	3 7	7.5	0.1	0 1	0	0.3
Blood	3 0	0.8	0	1.1	0	0
Colon	1.5	0.5	3.5	0.25	2.0	3.6
Small intestine	0 4	0.7	2 5		0.25	1.3
Liver	0 7	0 33	0 75	0 2	1.1	1.7
Stomach	0.8	0.9	0	0 7	0	0
Muscle	1 2	0 4	0	0.5	0.2	0.05
Lungs	0	0 4	0 4	0 15	0	0
Heart	1.0	0 7	0	0 2	0	0
Brain	0 3	0 2	0	0 1	0	0
Spleen	7 6	2 3	0	6 1	0	0
Pancreas	3 5	1 5	0	1 2	0	0
Skin	0.45	2 2		3 3		
Omentum	0.9	3 2	0	0.6	0	0

* Levels are expressed as gamma of As_2O_3 per Gram of wet tissue.

also in the rat in the peritoneal cavity when the drug was introduced directly into this region.   The liver and the kidneys suffered the greatest damage, notably degeneration and necrosis, especially after intravenous application.

The dithio derivatives (C.C. 914 and 1037) were alike in producing less tissue reaction after single doses to the several species.   No gastric damage was noted, and less severe peritoneal reaction followed application to this cavity.   Necrosis of a milder degree was noted in the liver and kidneys.   Other tissues showed inconsequential effects.

Chronic, short-term toxicity tests in rats produced no appreciable damage to the gastro-enteric tract, and except for albuminoid degeneration of a reversible character, no other significant effects were produced.   Only one of six monkeys

given C.C. 1037, 50 mgm./kgm. for 30 daily doses in excess of therapeutic need, had very slight fatty infiltration of the liver. Two macaques given C.C. 914 in 30 and 50 mgm/kgm., respectively, for 30 daily doses had only slight fatty infiltration of the liver, and mild albuminoid degeneration of the convoluted tubules of the kidneys occurred in one animal.

SUMMARY

(1) Substitution of dithiocarboxymethyl or dithiocarboxyphenyl groups (in p-carbamidophenylarsenous acid) did not appreciably alter amebacidal activity, *in vitro* or *in vivo*.

(2) Toxicity and tolerance tests in mice, rats, rabbits and monkeys indicated that the dithiocarboxyphenyl derivative was the least toxic.

(3) Reaction of the gastric mucosa and peritoneum to the direct application of dithio derivatives was less severe than to the arsenoxide. The liver, lungs and kidneys were appreciably damaged after single toxic doses of the arsenoxide.

(4) Within the range of therapeutic activity, the short-term (11 day) chronic toxicity test in rats and the 30-day chronic tolerance test in monkeys resulted in minimal damage to tissues after the dithio derivatives.

(5) After carbarsone oxide (p-carbamidophenyl arsenous oxide), distribution studies revealed exceptionally high levels of arsenic in the bile, blood and urine, moderate amounts in the feces, and contents of the stomach and small intestine, and in the colon, kidneys and liver, and smaller amounts in the lungs, heart and spleen. Appreciable tissue levels persisted for at least 9 days in rats after chronic intragastric therapy.

(6) Dithio derivatives (C.C. 914 and 1037) also accumulated in large amounts in bile, feces, urine, liver, kidneys, cecum and colon, with moderate amounts in contents of the stomach, cecum, and small intestine, blood, lungs, heart and spleen, and small amounts in brain, pleural and peritoneal fluids and thymus. The levels attained in these tissues in rats and rabbits after short-term chronic toxicity tests, were appreciably higher than in monkeys, on the 30-day chronic tolerance test.

(7) As with other arsenoxides, the arsenic of carbarsone oxide and its dithio derivatives, was bound by tissues roughly in proportion to the toxicity of each drug. Higher concentrations in intestinal tissue and in bile have been reported following injection of phenyl arsenoxides than after proportional doses of arsonic acids (10). This observation was confirmed and is believed to be significant.

(8) The suggestion of Voegtlin (2) that protozoa contain and are dependent upon smaller absolute amounts of -SH groups than mammalian tissue cells agrees with the experimental evidence presented.

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A DIURETIC FACTOR PRESENT IN NORMAL DOG, HUMAN, AND DIALYZED HUMAN URINE[1]

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During the course of some previous experiments it was noted that the repeated intravenous reinfusion of dog urine into anesthetized dogs resulted in a marked and progressive increase in the rate of urine flow (1). Since the diuresis so produced was considerably greater than that produced by a similar infusion of 0.9% NaCl, the observation was suggestive that dog urine contains some factor or factors which increase the rate of urine excretion.

The possibility that some urinary diuretic factor does exist has been tested by the infusion of dog and human urine and dialyzed human urine into normal unanesthetized dogs. When the rate of urine flow and total volume of urine excreted were compared with the rate and volume obtained following the injection of an appropriate control solution, it was found that a marked diuresis resulted, and that it could be attributed to the presence of some diuretic factor in the urine or dialyzed urine.

PROCEDURE. Female dogs were prepared for catheterization by an episiotomy. After they had recovered fully from the operation they were trained to lie on the animal board with loose restraining thongs. During the course of the experiments the animals received the stock dog diet. Water was removed from the cages from 12 to 16 hours before each experiment, and the animals received neither food nor water during the experiment. The animals remained in good health and showed no evidence of any permanent ill effects resulting from the infusion.

Throughout the experiment the urine was collected continuously, and its volume was measured at 15 minute intervals. The control rate of urine flow was determined for from 45 to 90 minutes. After the control rate of flow had remained relatively constant for at least forty-five minutes the animals received an intravenous infusion of either urine or the control fluid. The volume of the infusion was equivalent to either 0.5 or 1.0% of the body weight. It was necessary to give the infusion over a period of 2-5 minutes, since in a few instances, when urine was injected at a faster rate, the animal went into a state of collapse and died. Following the infusion, the rate of urine flow and total urine volume excreted were determined at approximately 15 minute intervals during continuous catheterization. These measurements were continued until the rate of urine flow had returned to or closely approximated the control rate and had remained at this level for at least 30 minutes.

The dog urine was obtained from donors by catheterization an hour or two before the infusion time, and the human urine was an over-night specimen voided before breakfast on the morning of the experiment. The donors of human urine were entirely free of any evidence of disease. The urine specimens were adjusted to a pH of 7.4 by the addition of an appropriate volume of 1 N NaOH, which was calculated from the titratable acidity data obtained on a sample of the specimen. The urea concentration (2) and the chloride con-

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[1] This paper was presented in part before the American Physiological Society. Chicago, Ill., 1947. Fed. Proc. 6: 154, 1947.

centration (3) were determined on aliquots of the adjusted specimen. The control solutions for the urine experiments were made by adding sufficient urea and sodium chloride to distilled water so that the final concentrations of chloride and urea were equal to those of the appropriate urine specimen.

TABLE 1

The effect of the infusion of dog and human urine and of dialyzed human urine on the rate of urine flow in the dog

C = Infusion of control solution   E = Infusion of urine or dialyzed urine.

EXP. NO.	WT.	VOL. INFUSED FLUID (VI)	AVERAGE CONTROL URINE FLOW (CF)		URINE FLOW AT MAXIMUM DIURESIS (MF)		RATIO OF MAX. FLOW TO CONTROL FLOW $\left(DR = \dfrac{MF}{CF}\right)$		TIME OF MAXIMUM DIURESIS	
			C	E	C	E	C	E	C	E
	kg.	cc	cc/min.	cc/min.	cc./min.	cc./min.			hours	hours
Dog urine										
1	6.0	60	0.17	0.07	0.68	1.54	4.0	22.0	1.00	0.50
2	8.3	80	0.19	0.07	1.00	1.24	5.3	17.7	1.25	1.25
3	7.8	40	0.08	0.05	0.50	0.93	6.3	18.6	0.50	0.75
4	8.0	40	0.09	0.07	0.76	0.97	8.4	13.8	1.50	1.00
5	8.0	40	0.05	0 05	0.35	0.79	7.0	15.8	0.25	1.50
6	5.0	25	0.07	0.05	0.37	0.93	5.3	18.6	2.25	0.50
Mean . .			0.11	0.06	0.61	1.07	6.1	17.8	1.13	0.92
Human urine										
7	7 8	40	0.13	0.06	0.75	1.51	5.8	25.2	3.25	1.25
8	7.0	40	0.13	0.19	0.48	0.93	3.7	4.9	2.00	0.30
9	7.8	40	0.09	0.14	0.45	1.03	5.0	7.4	1.25	1.50
10	8.3	40	0.06	0.11	0.64	1.33	10.7	12.1	1.50	2.25
11	5.0	25	0.04	0.07	0.09	0.61	2.2	8.7	1.00	1.25
Mean			0.09	0.11	0.48	1.08	5.4	11.6	1.80	1.31
Dialyzed human urine										
12	8.6	40	0.25	0.11	0.26	1.47	1.0	13.4	0.25	1.50
13	5.1	25	0.05	0.16	0.08	1.07	1.6	6.7	3.00	1.25
14	8.5	40	0.10	0.14	0.11	1.60	1.1	11.4	1.75	1.25
15	5.0	25	0.06	0.10	0.07	0.93	1.2	9.3	2.50	1.25
16	7.5	40	0.15	0.16	0.40	1.67	2.7	10.4	1.50	1.00
17	4.0	25	0.12	0.24	0.20	1.20	1.7	5.0	2.75	1.75
Mean			0.12	0.15	0.18	1.32	1.6	9.4	1.95	1.33

The dialyzed human urine was obtained by dialyzing in cellophane bags in the refrigerator freshly voided human urine (100–150 cc.) against 4 l. of distilled water for 3 days. The water was changed 6 times during the dialyses. At the end of this time the fluid in the bag was only slightly colored, was free of chloride ions, was neutral to litmus and it had a specific gravity of 1.001. The control fluid for these experiments was distilled water. Neither the infusion of distilled water nor of dialyzed urine was accompanied by gross evidence of hemoglobinuria

RESULTS. The effect of the infusion of experimental fluids (dog and human urine, and dialyzed human urine) and of the control fluids (see table 2) on the rate of urine flow is shown in table 1. It will be seen that at the time of maximum diuresis the mean rate of urine flow with dog urine was 1.7 times that ob-

TABLE 2

The effect of the infusion of dog and human urine and of dialyzed human urine on the volume of urine flow in the dog. Symbols some as in table 1

EXP. NO.	VOL. INFUSED FLUID (VI)	FLUID RECOVERED CORRECTED FOR CONTROL RATE (U)		RATIO RR = $\frac{U}{VI}$		DURATION OF DIURESIS		COMPOSITION OF CONTROL FLUID	
		C	E	C	E	C	E	%	%
								NaCl	Urea
	cc.	cc.	cc.			hours	hours		
Dog urine									
1	60	39	249	0 7	4.2	2.50	7.75	0.22	4.20
2	80	95	165	1.2	2.1	4.50	6.00	0.85	2 19
3	40	54	144	1 4	3.6	5.50	5.75	1.56	3.44
4	40	18	119	0 5	3 0	3.75	6.25	0.63	1.97
5	40	44	114	1.1	2 9	4.25	6.50	1.41	4.00
6	25	22	88	0 9	3 5	3.00	6.50	1.41	4.00
Mean		45	146	0.97	3.2	3 92	6.46		
Human urine									
7	40	77	124	1.9	3.1	6.00	5 00	0.55	1.57
8	40	34	52	0.9	1.3	5.00	4.00	0.55	1 57
9	40	32	84	0.8	2.1	4.25	3.50	0.72	1.14
10	40	38	106	1.0	2.7	2 50	4 75	0 61	1.09
11	25	3	67	0.1	2.7		5 75	0 61	1.09
Mean		37	87	0.94	2.4	4.44	4 60		
Dialyzed human urine									
12	40	0	154	0	3.9		6.25	*	
13	25	0	43	0	1.7		4 00		
14	40	0	129	0	3 2		4 00		
15	25	0	47	0	1 9		3 50		
16	40	7	122	0 2	3 1		4 50		
17	25	0	50	0	2 0		1.50	.	
Mean			91		2.6		3.95		

* Distilled water

tained with the control solution; with human urine the rate was 2.3 times that obtained with the control solution; and with dialyzed human urine the rate was 7.1 times that obtained with distilled water.

The increase in the rate of urine flow following the infusion of the various fluids has been calculated as the diuretic ratio (DR), which is the ratio of the

maximum rate of urine flow (MF) following the infusion of the experimental fluid or the control fluid to the control rate of urine flow (CF) obtained before the infusion. It will be seen that following dog urine the mean diuretic ratio was approxmately 3 times that for the control solution; following human urine the mean diuretic ratio was approximately 2 times that for the control solution; and following dialyzed human urine the mean diuretic ratio was approximately 6 times that obtained with distilled water. The lapse of time between the in-
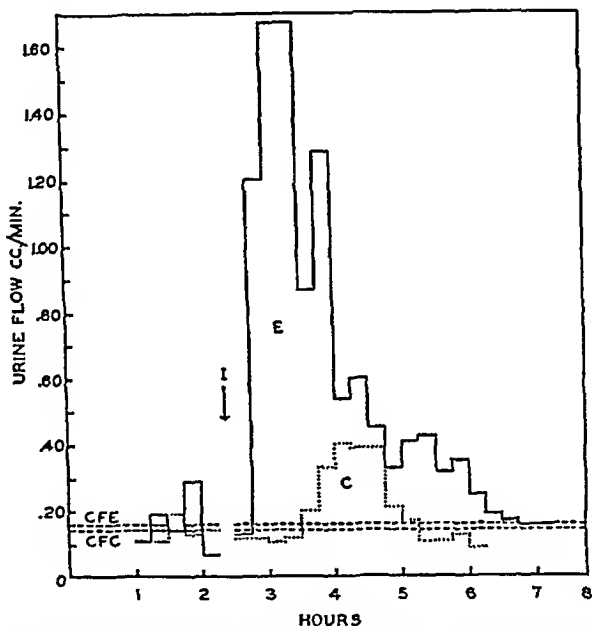

Fig. 1. The Diuretic Effect of Dialyzed Human Urine in the Dog (exp. 16). CFE = average control rate of urine flow for E; CFC = average control rate of urine flow for C; E = received 40 cc. dialyzed human urine; C = received 40 cc. distilled water; and I = point of fluid infusion.

fusion and the appearance of maximum diuresis was somewhat shorter with the experimental fluids than with the control fluids.

The effect of the infusion of the experimental and control fluids on the total volume of urine flow during the perod of diuresis is shown in table 2. The volume of fluid recovered as urine in excess of that which might have been expected, on the assumption that the control rate of urine flow would have persisted through the experiment had no fluid been infused, was considerably greater following the infusion of the experimental fluid than it was following the infusion of the control fluid. The above assumption was tested in several experiments and was found to be valid. In 5 of the 6 experiments in which distilled water was infused, no excess volume of fluid was recovered. The mean recovery ratio

$\left(RR = \dfrac{\text{volume of excess fluid recovered}}{\text{volume of fluid infused}}\right)$ was found to be approximately 1.0 for both types of control solutions and zero for distilled water. However, for dog and human urine and for dialyzed human urine, RR was respectively: 3.2, 2.4, and 2.6.

The mean duration of diuresis following dog urine was almost twice that for the control solution; the duration after human urine was approximately the same as that of the control.

The changes in the rate of urine flow following the infusion of dialyzed human urine and distilled water for one experiment (no. 16) are shown in figure 1. The mean control rates of urine flow were almost the same for the two experiments The increase in the rate of urine flow following dialyzed human urine occurred sooner, attained a much higher level and persisted for a longer time than was the case following the infusion of distilled water.

DISCUSSION. In considering possible explanations for the apparent increase in the rate of urine flow resulting from the reinfusion of dog's urine (1) it was thought that it might be attributed to the volume of fluid infused or to some small organic molecule or to inorganic ions present in the urine. The most obvious constituents of urine which might result in diuresis were thought to be urea and the chloride ion with its accompanying cation. Another possibility which was considered, but was thought to be an unlikely cause, was the excess of [H+] in the urine. As a result of these considerations, in each case, the control solution was made so that it contained the same urea and chloride concentration as found for the corresponding urine. For the same reason the urine was adjusted to pH 7.4 before it was infused.

It is apparent from these experiments that both dog and human urine and dialyzed human urine contain a substance which not only increases the rate of urine flow when infused, but which also decreases the total body water of the animal. This is shown by the observations that the mean fluid recovery ratio varied from 2.4 to 3.2 for the three experimental fluids, while it was approximately 1.0 for the two control solutions. Since in all of these experiments but two, the volume of fluid infused was equivalent to 0.5% of the body weight, the excess fluid recovered represents a reduction in total body water equivalent to approximately 1–1.5% of the animal's body weight.

An occasional dog urine, and human urine from one subject, resulted in an anti-diuretic response. This was presumably due to the presence of the pituitary anti-diuretic hormone. It is surprising that this result was not more frequently obtained since no attempt was made to produce hydration of the donor. The specific gravity of the infused dog urine especially was usually well above 1.020.

The nature of the diuretic substance, its source, and the mechanism of diuresis must await further experimentation. It seems clear from the experiments with dialyzed human urine that the substance has a rather large molecular size, and that its concentration in urine is rather small. It is not yet possible to say whether or not the diuretic substance is identical with the hypotensive factor

shown to be present in normal urine (4). Since the diuretic substance was present in urine obtained from normal animals and humans it would appear to be a physiologic substance.

SUMMARY

1. It has been demonstrated that when normal human and dog urine and dialyzed human urine are infused intravenously into normal unanesthetized dogs, in amounts equivalent to 0.5–1.0% of the body weight, there results a marked increase in the rate of urine flow over the pre-injection rate. The volume of urine recovered in excess of that infused plus that which would have been excreted without diuresis represents a reduction in total body water of the animal equivalent to approximately 1.0–1.5% of the body weight.

2. The infusion of an identical volume of control solution, which had the same sodium chloride and urea concentration as the corresponding urine, resulted in an increased rate of urine flow which was considerably less than that resulting from the urine infusion. The volume of urine recovered in excess of that which would have been excreted without infusion was equal to the volume infused. The infusion of the control solution did not decrease the total body water of the animal.

3. The infusion of a volume of distilled water equal to the volume of infused dialyzed human urine resulted in a slight increase in the rate of urine flow in one experiment. In five experiments the rate of urine flow was unaffected. The total body water of the animal was increased by the infusion of this volume of distilled water.

4. It is concluded that normal human and dog urine contain a non-dialyzable diuretic substance.

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DIGITALIS VI, PHARMACODYNAMIC STUDIES OF GITOGENIN*

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In recent years digitoxin has been extracted in unprecedentedly large quantities from the leaves of *digitalis purpurea* for use as an almost chemically pure therapeutic agent. In the purification of digitoxin, one of us (F.O.L.) obtained a white crystalline product which did not appear to be any of the well known cardiac glycosides or a saponin (digitonin) of digitalis. Owing to our interest in the color reactions (1, 2) of the glycosides of digitalis we decided to study this principle chemically and pharmacodynamically to extend our research program of further correlating the biological with the chemical assay, in order to establish the reliability of the latter.

Physical and Chemical Properties. The physical and chemical properties of this principle indicated (2) that it is gitogenin, a sapogenin named and described by Windaus and Schneckenburger (3), and further characterized by Windaus and coworkers (4) and Bouchet and Dugue (5). As noted by these authors (3) our principle also closely resembles digin described by Tambach (6). The salient properties of identification are as follows: Elementary analysis[1] showed: C—74.25 and 74.61%; H—10.03 and 9.86% with a molecular weight range of 350 to 387 (Rast camphor method). It is insoluble in water, dilute acids and dilute solutions of alkali; it is only slightly soluble in acetone and ether, but is soluble in alcohol and in chloroform. It is optically active, $[\alpha]_D^{25°}$ = −67.3° (c = 0.26, CHCl₃, 20 cm.), −64.4° (c = 1.18). Methanol solution (0.25%) shows only a general absorption in the 2300–3200 A° region.

After a partial sublimation between 210–220° C, the compound melts without decomposition to a colorless liquid, which on cooling congeals and remelts at the temperature observed for the product, 270–272° C (recrystallized from chloroform).

The absence of carboxyl, anhydride, ester or lactone groups and carbohydrate was shown by qualifying tests. Keller-Killiani, Legal, Baljet and Chen's (7) modification of Raymond (8) reactions were negative. The identity was further established by the melting points of the following derivatives: diacetyl derivative of the principle, M.P. 243–245°; diacetylgitogenin, 243–244° C (4); dipropionyl derivative, M.P. 193–196° C; dipropionylgitogenin, 195–196° C (4).

PHARMACODYNAMIC STUDIES. *Acute toxicity (White Rat).* Digitoxin and gitogenin respectively were injected intraperitoneally into the rat in 20 per cent alcohol containing 0.5 per cent of tragacanth. The concentration was 2 mg./cc. of each substance. The approximate LD_{50} of digitoxin after 24 hours under these conditions, 32 rats, was found to be 10 mg./kg. With gitogenin, using

28 animals, not any of the rats succumbed with 80 mg./kg., and in these concentrations the narcotic effect of the alcohol became noticeable.

Six rats 150 to 200 Gm. were given 80 mg./kg. of gitogenin by stomach tube dissolved according to the foregoing description. No effects were observed.

Perfused Frog's Heart. The insolubility of gitogenin in water made these experiments difficult. When alcohol was used to increase the solubility in Howell-Ringer's solution, no cardiac effect was observed that could not be produced by the concentrations of alcohol alone in the perfusate.

Several milligrams of gitogenin were reduced to an impalpable powder and agitated with Howell-Ringer's solution. This was perfused through the frog's heart without producing any effect. A similar suspension of digitoxin produced almost instantaneous cardiac stoppage.

Thus we are led to the conclusion that gitogenin is without effect on the perfused frog's heart, which may be either due to its insolubility or its inherent cardiac inertness. This confirms the findings of Gottlieb (through Tambach) (6) on the frog.

Electrocardiographic Studies (Dog). Under light ether anesthesia five dogs were given gitogenin intravenously and electrocardiographic tracings were taken at 5 minute intervals. The principle was injected in 20 per cent alcohol and solution made isotonic with sodium chloride containing 0.5 per cent tragacanth to prevent the separation of the compound. The concentration was 40 mg./100 cc. and the rate of injection was 20 cc. in 5 minutes. No typical digotoxin effects were observed. Large volumes of solution were required to produce cardiac irregularities and these were preceded by marked respiratory depression owing to the large volume of alcohol injected. The changes could be produced in control dogs by the injection of the alcohol tragacanth mixture in large volumes.

Cat Assay. We attempted to determine the lethal dose of gitogenin for the cat using a modified U. S. P. procedure.

It was dissolved in 5 cc. of dehydrated ethanol and mixed with 95 cc. of normal saline solution. The finely divided precipitated gitogenin was kept uniformly suspended by intermittent agitation. The concentration was 0.4 mg./cc. and the alcohol only 5 per cent. Twenty cubic centimeters were injected during the first 5 minutes and 1 cc. a minute thereafter. Electrocardiographic tracings were taken each 5 minutes. In 3 animals no concordant data were obtained. No lethal dose could be obtained in one animal. In the other 2 animals the fatal dose was approximately 8 mg./Kg.; the respiration ceased before cardiac stoppage. In the cat, as in the dog, no typical digitoxin effect could be detected electrocardiographically.

Baljet Reaction and Gitogenin. As gitogenin is obtained in the purification process of digitoxin, it generally occurs in impure digitoxin. It is important to observe that this compound, devoid of cardiotonic activity, exerts no influence on the Baljet reagent. Therefore in this chemical method of assay, now official in the Pharmacopoeia, it will not be estimated as digitoxin. This statement holds also for the Raymond reaction (8) and the Keller-Killiani (2) reaction.

CONCLUSIONS

1. Gitogenin has been obtained in quantity as a by-product in the purification of digitoxin.

2. Pharmacodynamic studies show it to be devoid of typical cardiotonic activity.

3. Gitogenin does not give a positive reaction with the Baljet reagent and other characteristic color reactions.

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THE ACUTE JOINT TOXICITY OF ATABRINE, QUININE, HYDROXYETHYLAPOCUPREINE, PAMAQUINE AND PENTAQUINE*

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In the treatment of malaria, atabrine, quinine and pamaquine have been employed jointly with the supposition that a combination of these may be more effective than the individual drugs in the cure of the disease. A study of the joint toxic action is, therefore, of practical importance in regard to their joint toxicity in the host as well as to the efficacy of the combination of these drugs in malarial therapy.

The present investigation was undertaken with these objectives in mind. The joint toxic effects of atabrine, quinine, hydroxyethylapocupreine, pamaquine and pentaquine, were studied in mice by the dosage-mortality relationships laid down by Bliss in respect to the toxicity of poisons administered jointly (1).

EXPERIMENTAL. Male mice, 2 to 3 months of age, weighing between 18 to 26 grams, were used. Atabrine·2 HCl, quinine·HCl, pamaquine·HI, hydroxyethylapocupreine·2 HCl, pentaquine·H_3PO_4 were dissolved in water at a concentration of 0.10 ml. per 10 gram body weight and administered intraperitoneally into a group of 20 mice for each dose. The dosage was calculated in terms of the base of the compound. The animals were kept under observation for two days; the number of deaths were recorded at the end of the period. It was found that there was no difference in combined mortality whether the two drugs were given in one solution or in separate solutions.

RESULTS AND DISCUSSION. The percentage of mortality was calculated according to the method of Reed and Muench (2). The dose-mortality relationship will be presented in terms of logarithms and probits by the equation $y = a + b \log x$: y = probit, x = dose, a and b = constants (1).

A. *Atabrine and Quinine·* The dosage-mortality curves of atabrine (A), quinine (Q), atabrine and quinine (A + Q) are given in figure I. Two significant features which may be pointed out are: (a) The similarity in the slope of the lines and (b) the magnitude of LD_{50} (at probit 5) which is about the same for atabrine, quinine and the mixture. The latter finding is rather accidental due to the fact that the LD_{50} of atabrine is nearly equal to that of quinine. The LD_{50} of atabrine and of quinine were found in six experiments on different days to be: A = 160, 167, 180; Q = 170, 178, 174 mgm./kg. In this particular experiment in which the mixture was one of equal parts of the two drugs, the LD_{50} (174 mgm./kg.) consisted of half of the LD_{50} dose of atabrine and half of that of quinine. Since one half of the LD_{50} dose of either drug is non-lethal to mice, it means that, toxicologically, the two drugs act independently and in a similar manner. This is also indicated by the dosage-mortality curve of the mixture as that for the individual drug alone when formulated as follows:

$$Y_{A+Q} = a + b \log (D_A + KD_Q) \text{ where } D_A \text{ and } D_Q \text{ are individual doses of atabrine and}$$

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quinine respectively in the mixture and K the ratio of their equi-toxic doses such as LD_{50} (1).

B. *Quinine and Hydroxyethylapocupreine:* To elucidate further the joint toxicity of drugs which act independently and similarly, our data of the combined toxicity of quinine (Q) and hydroxyethylapocupreine (H) are included in figure I. In this case, hydroxyethylapocupreine (LD_{50} = 345 mgm./kg.) was found to be about $\frac{1}{2}$ as toxic as quinine (LD_{50} = 173 mgm./kg.) The mixture was made of $\frac{2}{7}$ (Q) and $\frac{3}{7}$ (H) in terms of their $LD_{50}s$; the theoretical value of LD_{50} for this mixture would be 278 mgm./kg. consisting of 71 mgm. (Q) plus 207 mgm. (H), on the basis that the two drugs act independently and similarly. The experimental values were 68 mgm. (Q) and 194 mgm. (H) per kilo.
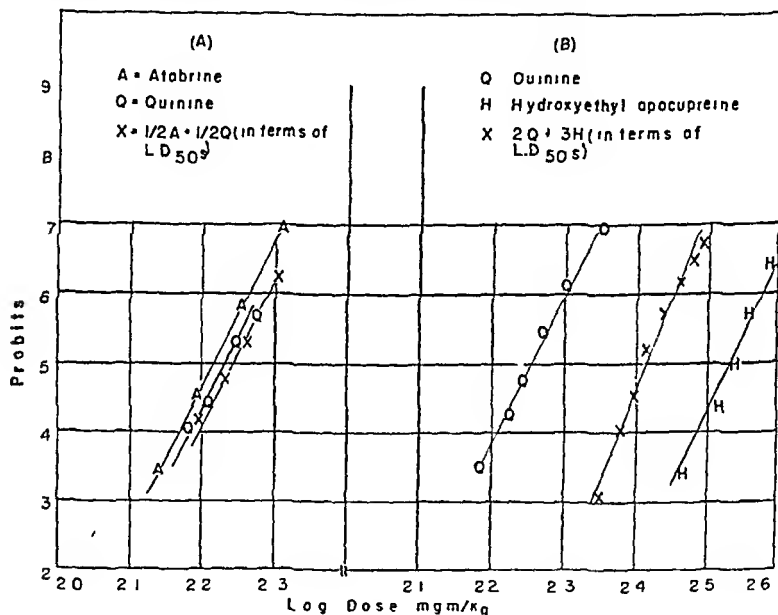

Fig. I

C. *Atabrine and Pamaquine:* As indicated by graphs in figure II (a), the lethal effect of pamaquine (LD_{50} = 17 2 mgm./kg.) is not influenced by a sublethal dose of atabrine (100-140 mgm./kg.) This signifies that atabrine and pamaquine act independently but diversely. To see whether or not there is a correlation in susceptibility to the two drugs, the following mixtures of atabrine and pamaquine, 2A + 3P, A + P, 3A + 2P, in terms of their $LD_{50}s$ were investigated (1) The results are graphically presented in figure 2 (b, c, d respectively). Included in the figure are also graphs drawn from values for independent and diverse action, computed from the equation, $Pc = P_P + P_A (1 - P_P) (1 - \gamma)$ by letting γ equal to zero. Pc is the proportion of animals killed by the combination of two drugs when P_P and P_A are the proportions of animals dying from the same amount of the two drugs administered alone and γ the coefficient of association The experimental curves coincide well with the theoretical when the equi-toxic concentration is equal or less than that of pamaquine (2A + 3P, A + P) With the increased relative amount of atabrine and above the sublethal level as in 3A + 2P and A + P, the combined mortality is greater than the

expected. Similar results were obtained in 3 separate experiments. A discussion of this difference in comhined mortality will be taken up subsequently.

D. *Quinine and Pamaquine:* Preliminary experimentation revealed that a mixture of quinine and pamaquine produces a mortality of mice much greater than what can be accounted for hy a joint toxicity which is due to an independent and similar action of tbe two drugs. For instance the LD_{50}s of quinine (Q) alone and pamaquine (P) alone are 176 and 17.6 mgm./kg. respectively (Figure III). The LD_{50} of a mixture of the two consisting of 2Q + 3P in terms of their LD_{50}s was found to be 47.8 mgm. Q plus 8.2 mgm. P, much less than the calculated values of 70 mgm. of quinine and 15.6 mgm. of pamaquine for a joint toxicity due to tbeir similar action. Furthermore, the dosage-mortality curve of the joint toxicity appears to be tbat for a different drug. Experiments were tbus designed to investigate the
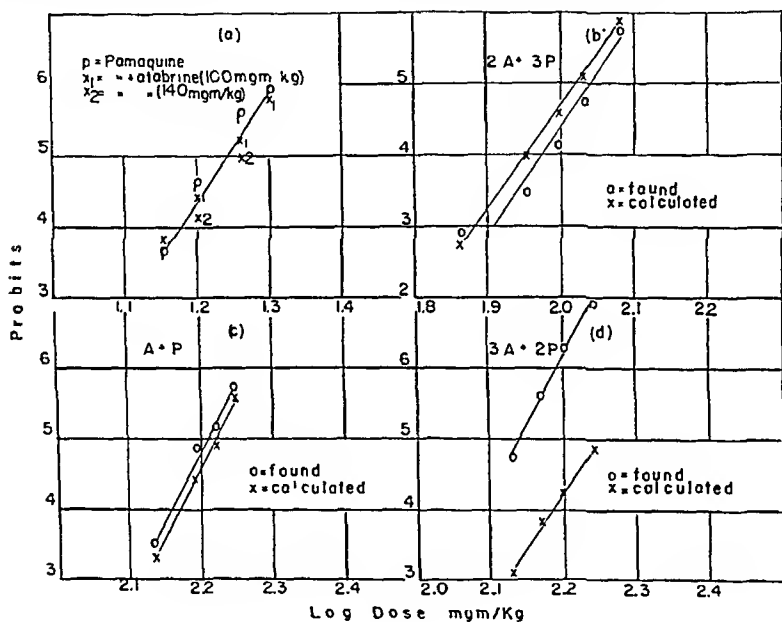

FIG. II

synergistic nature of their toxic action following the procedure given hy Bliss with the equation: $Log (A + K) + i \log B = Log J$ or $(1 + k_1A)B^1 = k_2$ where A and B are doses of the two drugs in the mixture; K, i, k_1, k_2, are constants.

The dosage-mortality curves in figure IV were ohtained with various doses of pamaquine to each of which a sublethal dose of quinine was added. The intersection of the horizontal line at probit 5 gives the amounts of quinine and pamaquine in a mixture to produce a joint toxicity, a mortality of 50 per cent (Table I). The curve in figure V was constructed hy plotting the logarithms of doses of quinine and pamaquine in a mixture that will kill 50 per cent of mice. The corresponding straight line was ohtained hy assigning the desired value of K to log (A + K) where A is the dosage of quinine and plotting the Log (A +K) against log B, B being the concentration of pamaquine. The computed values for i, k_1, and k_2 are 2, $\frac{1}{56}$, 250 respectively. By the terminology of Bliss, the intensity of synergism of quinine and pamaquine is 12.5 (k_1k_2).

E. *Quinine and Pentaquine:* In view of the similarity in chemical structure between pamaquine and pentaquine, the latter was also investigated for its synergistic joint toxicity with quinine. The LD_{50} values of mixtures of quinine and pentaquine are given in table I. Their joint toxicities are synergistic in nature. The calculated values for the constants, i, k_1, and k_2, are 1.2, $\frac{1}{26}$ and 107 respectively. The intensity of synergism of quinine and pen-
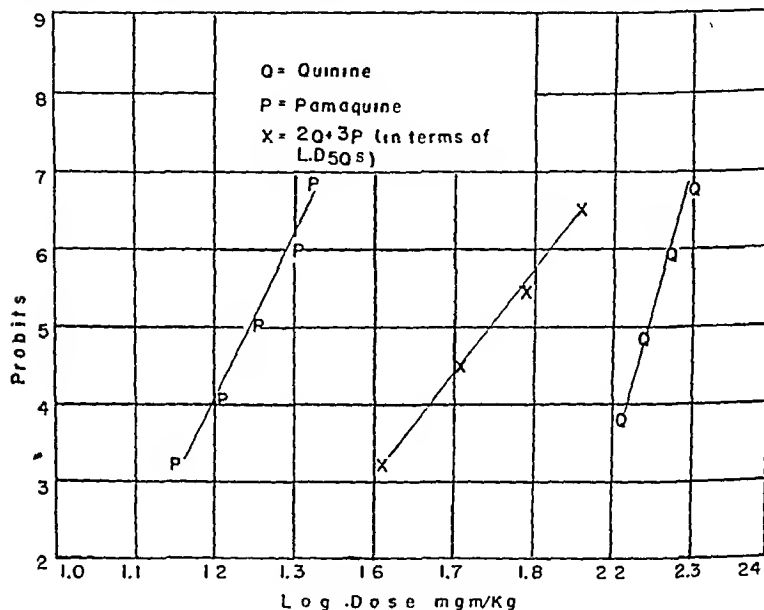

Q = Quinine

P = Pamaquine

X = 2Q+3P (in terms of L.D50 s)

Probits

Log . Dose mgm/Kg
Fig III

TABLE 1

The $LD_{50}s$ of mixtures of quinine and pamaquine (A), quinine and pentaquine (B)

(A)		(B)	
Quinine mgm /kg	Pamaquine mgm./kg	Quinine mgm /kg	Pentaquine mgm./kg
0	17 90	0	45.7
10	13 00	5	42.6
20	11 30	10	36 1
40	9 73	20	27 6
80	7 09	40	21 9
150	5 64	80	11.2

taquine is thus 5.35 ($k_1 k_2$), which is about 0.4 of that (12.5) for quinine and pamaquine. Since pentaquine is about 0.4 as toxic as pamaquine, their joint toxicity with the same quantity of quinine is, in terms of their equitoxic doses, about the same

DISCUSSION. A similarity in the acute toxic action of atabrine and of quinine is indicated by the symptoms developed in animals soon after injection. Mice

receiving atabrine suddenly develop tremors and convulsions and die within one hour after the administration of the drug. Similar symptoms are observed following the injection of quinine. A common site of action may be accounted for the joint toxicity of atabrine and quinine.

Symptomatically, the principal toxic effect of pamaquine is that of depression. The animal becomes quiet, somnolent and dies in a depressed state $\frac{1}{2}$ to 2 days after receiving the drug. The independent and diverse toxic actions of atabrine and pamaquine are thus also indicated by the difference in acute toxic symptoms. It remains to be investigated as to why the combined mortality

Fig. IV
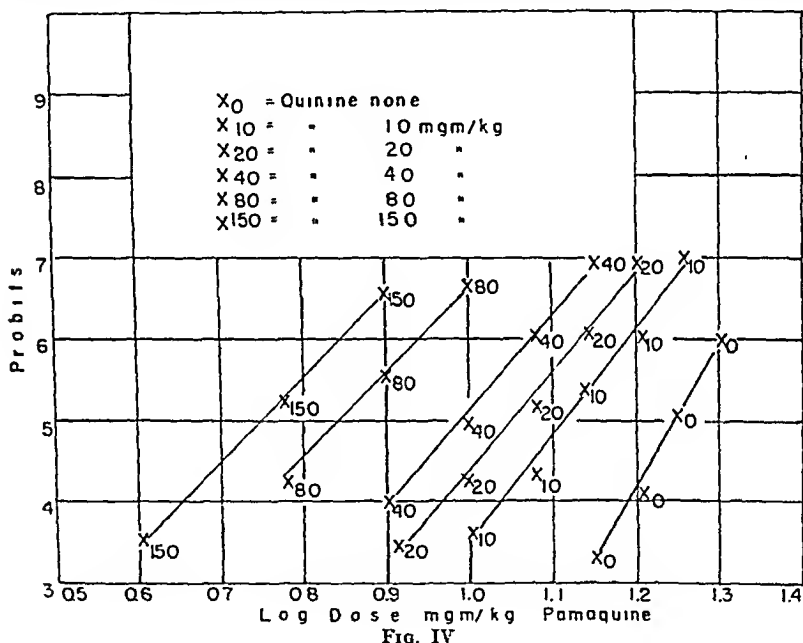
due to atabrine and pamaquine is greater than the expected value for independent and diverse action when the amount of atabrine in the mixture is in the range of lethal concentrations. It is possible that the rate of elimination of atabrine in the animal is decreased as a result of the toxic effects of pamaquine. This decrease in the rate of elimination of atabrine appears to be not consequential insofar as death of the animal is concerned when the initial concentration is low. However, it will be a contributing factor to mortality when the initial concentration of atabrine in the animal is high.

Similar phenomena were observed in our other toxicity studies where the inhibition of growth of young rats by a drug was taken as an index for toxicity (3). The effect on growth by a combination of atabrine and pamaquine may be attributed to the toxic effect of one drug alone when the dosage of the other

is below the toxic level. When both are at toxic levels, however, a greater inhibition was observed for a mixture of the two than what can be counted for by an additive effect of the individual drugs.

It should be pointed out, here, that there may or may not be a similar correlation in the joint toxicity of drugs by the acute and by the chronic toxic effects.
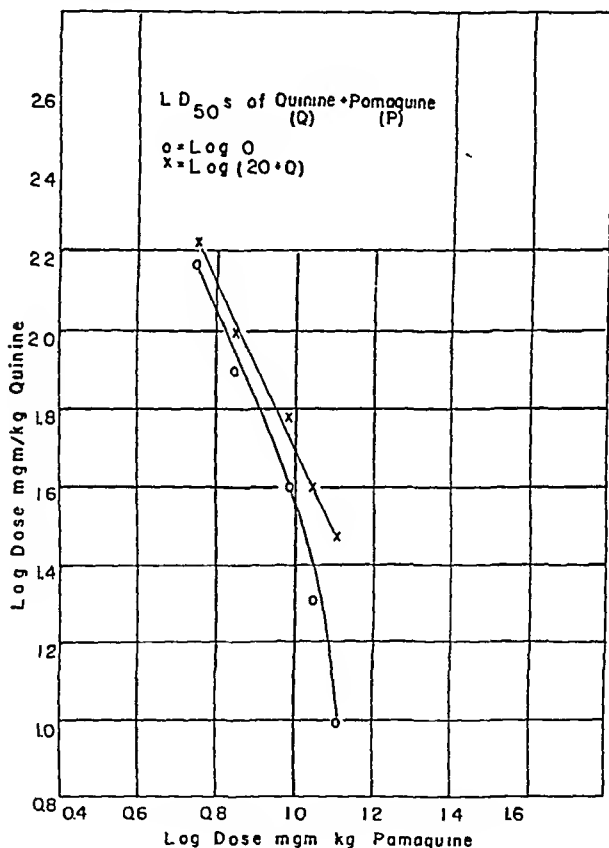

Fig. V

In the latter, the joint toxicity will to be complicated by factors which are due to some anatomical lesions produced by the drug. In acute toxicity, on the other hand, the functional impairment of the vital organs is primarily concerned.

In the case of quinine and pamaquine when judged by the toxic symptoms produced by the individual drugs, the joint toxicity would not be different from that of a mixture of atabrine and pamaquine. Since only a very small amount of quinine, $\frac{1}{30}$ of the minimal lethal dose, is sufficient to reveal its synergistic

action with a minimal lethal dose of pamaquine in acute mortality, the site and the mechanism of this action of quinine are evidently different from those causing sudden death of animals with a lethal dose of quinine. With the present state of knowledge, we are not able to offer an explanation for the mechanism involved in the synergistic action of quinine and pamaquine. It may be speculated that quinine and pamaquine, or their degradation products, act in the different steps or on the different enzymes or substrates of an enzymatic process which is essential for life. In our recent unpublished observations, pamaquine was found to inhibit the enzymatic activity which destroys quinine, but so did atabrine.

Quinine and pamaquine, quinine and pentaquine have also been found to be synergistic in their therapeutic effects in bird malaria. These experiments will be reported in a separate communication.

SUMMARY

The acute lethal toxicities of atabrine and quinine, quinine and hydroxyethylapocupreine, atabrine and pamaquine, quinine and pamaquine, quinine and pentaquine, were investigated by intraperitoneal injection in mice. It was found that the combinations of these antimalarials are different in their joint toxicity: (1) Atabrine and quinine, quinine and hydroxyethylapocupreine, act independently and similarly (2) Atabrine in sublethal doses and pamaquine act independently but diversely. (3) Quinine and pamaquine, quinine and pentaquine are synergistic in their joint action.

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AN EVALUATION OF ASSAY METHODS FOR ARSENICALS

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Recently, an in vivo method and an in vitro method have been developed by us for the assay of the trypanocidal potency of antimonials. In the former the increase of survival time of mice infected with T. equiperdum is taken as a measure of the trypanocidal activity of a substance. It gives results similar to those obtained by a curative test. In the latter test (our in vitro method) the effect of antitrypanosome substances on the lowering of glucose metabolism of suspensions of the parasites is used to determine their activity. The relative antitrypanosome potencies as determined by this procedure agree with those obtained by assays in vivo for the trivalent but not for the pentavalent organic antimony compounds (1, 2).

The purpose of the present investigation is to make an evaluation of the two methods for the assay of the trypanocidal potency of arsenicals.

MATERIALS AND METHODS. Male mice of a homogenous stock infected with T. equiperdum were used for in vivo assays. For in vitro testing, the parasites were collected from the blood of infected rats. The detailed procedures for these methods have been previously described (1, 2).

In addition, the following procedure was employed to determine the lethal concentrations of arsenicals on T. equiperdum in vitro. One part of fresh blood drawn with a blood pipette from the tail of an infected rat was diluted with 4 parts of a special Ringer-Locke solution containing the drug in the well of a hanging drop slide. After thorough mixing, the well was covered with a slip. At 5 minute intervals the lethal effect of the arsenical on trypanosomes was observed under the microscope with a minimal amount of the material. The death of around 95 per cent of the parasites was considered as the endpoint. The experiment was conducted for one hour at room temperature. The parasites will live in such preparations without drugs for at least three hours.

The Ringer-Locke solution contained 300 mgm per cent glucose, 500 mgm per cent sodium citrate and 10 per cent M/15 phosphate buffer at pH 7 5. In control experiments with this medium, the glucose metabolism of the trypanosomes was found to be proceeding linearly and there was no significant difference in parasite counts at the beginning and at the end of one hour (2).

RESULTS AND DISCUSSION. The data presented in tables 1 and 2 were obtained by in vivo and in vitro procedures respectively. In in-vivo assays, the trypanocidal activities of arsenicals were ascertained: (A) by the quantities of substances required to give a 50 per cent cure of the infected mice and (B) by the suppressive doses that increase the survival time of infected animals a 100 per cent (1). In in-vitro tests, potencies of the drugs were determined: (A) by the concentrations of arsenicals that produce a 50 per cent lowering of

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glucose metabolism of trypanosomes and (B) by the concentrations of the drugs that cause death of the parasites at the end of one hour.

It may be seen from the values of their relative potencies among Mapharsen, neoarsphenamine and tryparsamide that the suppressive and the curative method give practically the same results within the limits of experimental variations. The value obtained from the lowering of glucose metabolism agrees

TABLE 1

Assay of Arsenicals In Vivo

ARSENICAL	(A) CURATIVE METHOD				(B) SUPPRESSIVE METHOD	
	*C D_{50} Mgm / Kg	Range ± 2 S E	†C D $\frac{\text{C.D.}_{50}\text{X}}$	Range ± 2 S.E.	‡S S D Mgm /Kg. (Mean ± S E.)	$\frac{\text{S S D.M.}}{\text{S S.D X.}}$ ± 2 S E.
Mapharsen	4.17	3 82–4.55	1		0.76 ± 0.026	1.0
Neoarsphenamine	19.70	18.8–20.8	0.211	0.198 –0.225	3.72 ± 0.325	0.204 ± 0 038
Tryparsamide	1110	994–1240	0.0037	0.0034–0.0040	302 ± 7.37	0.0025 ± 0.0002

* C.D = Curative Dose

† M = Mapharsen

  X = Arsenical to be compared

‡ S S D = Standard Suppressive Dose, average of 3 experiments

TABLE 2

Assay of Arsenicals in Vitro

ARSENICAL	(A) INHIBITION OF GLUCOSE METABOLISM		(B) LETHAL EFFECT	
	*I C_{50} (Mean ± S E) γ or mgm /cc	**I C_{50}M $\frac{}{\text{I.C.}_{50}\text{X}}$ ± 2 S E	*L C_{50} (Mean ± S.E) γ/cc.	L C_{50}M $\frac{}{\text{L.C.}_{50}\text{X}}$ ± 2 S E
Mapharsen	4 2 ± 0 30 γ	1	2.3	1
Neoarsphenamine	16.7 ± 1 79 γ	0 251 ± 0 064	73 3	0.031 ± 0.007
Tryparsamide	23 3 ± 4.10 mgm	0.00018 ± 0.00007	(64 mgm./cc → no effect)	

* I C_{50} = Concentration for 50% inhibition of glucose metabolism (average of 3 experiments)

† L C = Lethal concentration for 60 minutes (average of 3 experiments).

‡ M = Mapharsen, X = Arsenical to be compared

with those determined by assays in vivo for the trivalent arsenic compounds; it is lower for tryparsamide. This is in agreement with the findings for antimonials. Where the potencies of Mapharsen and neoarsphenamine are compared by their lethal concentrations in vitro, the relative potency of the latter is much less than that obtained by the other methods. In the case of tryparsamide, as high as 64 mgm./cc does not show a lethal effect at the end of one hour. It is obvious that the in vitro methods are not applicable for the in vivo trypanocidal activity of the pentavalent forms such as tryparsamide. It should be mentioned, however, that other in vitro methods conducted under

different experimental conditions might give a different value of trypanocidal potency for tryparsamide (3, 4).

These observations bring forward the following pertinent questions: (A) How to explain the difference in values of the relative trypanocidal potencies as determined by the lowering of glucose metabolism and by the lethal effect in vitro? (B) How to account for the agreement in values assayed in vivo and
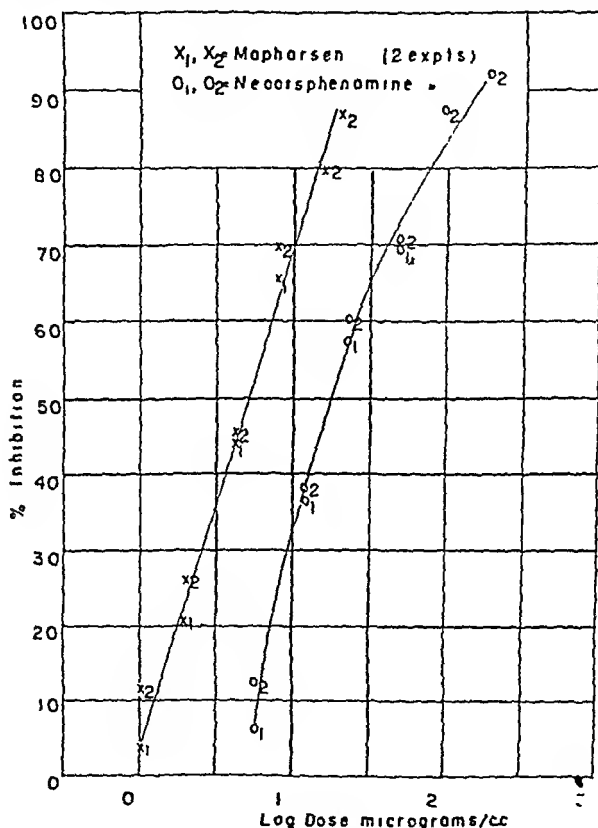

Fig. 1

in vitro by the lowering of glucose metabolism for the trivalent but not for the pentavalent arsenic compounds? and (C) How to interpret the higher trypanocidal potency of tryparsamide by assays in vivo than by assays in vitro? To answer the first two questions, the following explanation appears to be reasonable. In determining the antitrypanosome potencies of substances by the lowering of glucose metabolism, there are being measured both their toxic and lethal effects. In other words, it consists of a suppression of glucose metabolism of the living trypanosomes and a lowering of glucose utilization as a result of

the decrease in the number of parasites. Which factor will contribute more than the other in their total effect depends probably mainly upon the range between the toxic and the lethal doses of a compound.

As indicated by the curvature of the lines in figure 1 for the relation between the lowering of glucose metabolism and doses, the smaller the range between the toxic and the lethal dose, the more linear the curve becomes. The linear relationship in the case of Mapharsen suggests that the lowering of glucose metabolism here is primarily due to the decrease in the number of parasites. The curvature of the line for neoarsphenamine indicates a more pronounced toxic effect. The inclusion of a toxic factor in the determination by the lowering of glucose metabolism may account for the higher relative trypanocidal potency of neoarsphenamine as measured by this method than by the procedure where the assays were conducted using lethal concentrations.

A similar situation exists in vivo regarding the effect of drugs on trypanosomes. The parasites are destroyed either by the lethal action of the drug directly or, when intoxicated by it, by the defense mechanism of the body. In in-vivo assays of the trivalent arsenicals, undoubtedly both the toxic and the lethal effect are measured. This will explain the fact that the relative potencies of these compounds as determined by the lowering of glucose metabolism agree with those obtained by assays in vivo.

In the case of tryparsamide, on the other hand, the lowering of glucose metabolism of one hour is entirely due to its toxic effect on trypanosomes. The concentration of the drug which gives a 50 per cent suppression of glucose metabolism is not lethal to the parasites at the end of one hour (table 2). This lack of a lethal effect of tryparsamide in vitro, under the conditions of the experiment, accounts for the discrepancy in values of potency as determined by in vivo and in vitro procedures. Conversely, the greater the lethal action of a substance on trypanosomes, the better will be the agreement in values by in vivo and by in vitro assays.

SUMMARY AND CONCLUSION

In conclusion it may be stated that the suppressive method using the increase of survival time of infected mice as a measure of the trypanocidal activity is as accurate as the curative method for the assay of the relative trypanocidal potency of arsenicals. It is simpler and less time consuming than the latter procedure. For potent and quick-acting trypanocidal substances, the in vitro method based on the lowering of glucose metabolism by the antitrypanosome effect of drugs offers several advantages. Besides simplicity, rapidity, and the linear relationship between dose and effect, it gives results which are reproducible within narrower limits of experimental variations than those by other methods.

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A MOUSE ASSAY FOR CURARE

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This paper describes a simple, objective method for the assay of curare activity. The method utilizes mice as the test animals and employs the sloping, rotating cylinder apparatus recently described by Young and Lewis (1). So far as can be ascertained this method has advantages over other curare assays which employ the intact animal (2, 3), although none of these procedures are fully described in the literature. Curare assays involving the use of isolated nerve muscle preparations have been described (4).

The equipment used in the investigation was that described by Young and Lewis (1). Commercial samples of d-tubocurarine chloride[2] and Intocostrin were used as the active agents. In each experiment the active solution was diluted with distilled water to the required concentration and 0.25 cc. of the appropriate dilution was injected subcutaneously into each mouse. Female mice weighing from 15 to 17 grams were used in the study. Immediately after injection the mice were placed in the rotating cylinders and mice falling away from the cylinders during the first twenty minutes were considered as reactors. Experience showed that reactions rarely occurred later than twenty minutes from the time of injection.

On the basis of preliminary trials a range of dose levels was selected and two series of experiments were performed. Series A consisted of eleven experiments which were designed to examine the dosage-response relationship. In series A a solution of Intocostrin was employed as the active agent. Series B consisted of five experiments in which the contents of a vial of Intocostrin was compared, as regards potency, with the contents of a vial of Solution Tubocurarine Chloride.

In carrying out the experiments of Series A twenty-five mice were assigned at random to each of four dose levels. The dose levels were arranged in geometric progression. The maximum dose administered and the geometric ratio used in each experiment are indicated in table 1. After completion of an experiment the proportions of mice reacting at the different dose levels were transformed to probits and the slope of the logarithm-dosage response relationship was computed in the usual manner. The estimates of slope and E.D.$_{50}$ for

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[2] Solution Tubocurarine Chloride Squibb and Intocostrin were kindly supplied by E. R. Squibb of Canada, Limited. Solution Tubocurarine Chloride Squibb contains 2.7 mgm. of anhydrous crystalline d-tubocurarine chloride per cc and is said to have a potency of 20 units of curare activity per cc. as measured by the rabbit head-drop cross-over assay (3). Intocostrin is an extract physiologically assayed by the rabbit head-drop cross-over method to contain 20 units of curare activity per cc.

the individual experiments are exhibited in table 1. The estimated slopes were found to differ significantly from one experiment to another. The weighted average of the slopes was 7.81. The logarithm-dosage response curve did not depart from linearity in any experiment.

Series B consisted of five two-level assays. The assays were employed to compare the potencies of a sample of Intocostrin and a sample of Solution Tubocurarine Chloride. In conducting each assay mice were distributed at

TABLE 1

Results of experiments from series A

EXPERIMENT NUMBER	DATE OF EXPERIMENT	MAXIMUM DOSE IN CC. OF INTOCOSTRIN	RATIO OF DOSE CONCENTRATIONS	SLOPE	E D.50 IN CC. OF INTOCOSTRIN
1	8/4/47	0.0029	1.1	13.94	0.0027
2	9/4/47	0.0029	1.1	5.41	0.0031
3	11/4/47	0.0033	1.1	9.09	0.0029
4	14/4/47	0.0033	1.1	10.01	0.0026
5	15/4/47	0.0033	1.1	2.89	0.0029
6	16/4/47	0.0029	1.1	3.63	0.0025
7	17/4/47	0.0042	1.2	11.16	0.0029
8	18/4/47	0.0036	1.2	9.08	0.0021
9	25/4/47	0.0031	1.2	12.02	0.0022
10	26/4/47	0.0028	1.2	3.99	0.0019
11	28/4/47	0.0028	1.2	13.20	0.0017

TABLE 2

Results from Five Curare Assays

ASSAY NUMBER	CC. OF CURARE SOLUTION INJECTED/MOUSE		NUMBER OF MICE REACTING				POTENCY OF INTOCOSTRIN IN TERMS OF TUBOCURARINE -PER CENT	CONFIDENCE LIMITS P=0.95
	LOWER DOSE	HIGHER DOSE	SOLUTION TUBOCURARINE CHLORIDE		INTOCOSTRIN			
			S_1	S_2	U_1	U_2		
1	0.0018	0.0025	14/25	23/25	11/25	21/25	91	77-107
2	0.0016	0.0023	9/25	16/25	10/25	14/25	97	76-132
3	0.0016	0.0023	13/25	19/25	10/25	18/25	91	71-116
4	0.0016	0.0023	9/25	21/25	4/25	18/25	88	77-100
5	0.0016	0.0023	8/23	20/25	10/25	16/25	95	77-116

random into four groups of twenty-five each. Two doses of Solution Tubocurarine Chloride, S_1 and S_2, and two doses of Intocostrin, U_1 and U_2, were assigned at random to the groups. The Tubocurarine and Intocostrin were assumed to be equal in curare activity. The ratio of higher to lower dose (i.e. $S_2/S_1 = U_2/U_1$) was 1.4. The quantities of curare solution injected per mouse are given in table 2, together with the final estimates of potency and their confidence limits. The proportions of mice reacting to Tubocurarine and Intocostrin are presented in the table in order to illustrate the type of result which may be expected from curare assays carried out under the conditions described.

There was no indication from the usual χ^2 test that the estimates of potency from the various assays differed significantly. The pooled estimate of the relative potency of Intocostrin in terms of Solution Tubocurarine Chloride was 91% with confidence limits of 84 to 98% (P = 0.95). There was no evidence to indicate that the slope for Solution Tubocurarine Chloride differed significantly from the slope for Intocostrin. The estimated slopes varied from 3.78 to 10.04 in the five assays. The weighted average of the slope estimates for the five assays was 6.43.

The assay procedure as described was found to be simple, objective and inexpensive. An assay employing one hundred mice could be performed in approximately half an hour. The mortality among 1,600 mice used in the experiments was less than 6%.

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4. Chou, T. C. Br. J. Pharm. and Chemo. 2, 1. 1947.

FURTHER OBSERVATIONS ON THE PHARMACOLOGY OF 'DOLOPHINE' (METHADON, LILLY)[1]

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'Dolophine' (Methadon, Lilly), a new analgesic drug, has been analyzed for a number of pharmacological properties. Recently, this laboratory reported that the actions of this drug were similar in general to those of morphine, but that some differences were noted. However, further work was needed concerning details of its effects. In particular, there were the problems of tolerance, addiction, respiratory depression, effect on smooth muscle, and mechanism of action.

Although attempts previously had been made along some of these lines, the results were not necessarily final or conclusive. The following report deals mainly with animal work. In some cases, comparisons were made with demerol. The latter compound and 'Dolophine' were used as the hydrochloride salts, while the sulfate was employed in the case of morphine.

1. *Tolerance and addiction studies in dogs.* Since a constant dose of 'Dolophine' did not result in tolerance to its analgesic effect (1, 2), larger and more frequent amounts were tried. A total of 10 dogs were studied, the animals being divided into two groups. Group A consisted of 4 dogs observed for a 32-day period, while in Group B, 6 dogs received injections for 56 days.

In Group A, a dose of 5 mg. per kg. was injected intraperitoneally once daily for the first 4 days. Thereafter injections were made twice daily at 8:30 a.m. and 4:30 p.m. The dose was gradually increased to 20 mg. per kg., b.i.d., for the last 6 days. Only 2 dogs survived the entire test; one died on the fourteenth day and the other on the thirty-first day. Observations of analgesia, pulse, respiratory and nervous reactions to 'Dolophine' were made at the start of the trial period and weekly thereafter. As a standard dose, 5 mg. per kg. was used for these observations throughout. Analgesic effects were measured by the Andrews and Workman modification (3) of the Hardy, Wolff, Goodell procedure (4).

While on test, considerable loss of body weight occurred, owing apparently to poor appetite. Finally, feeding by stomach tube was instituted on the twenty-third day to prevent death from inanition.

Some of the results are shown in figure 1. Tolerance to the analgesic action of 'Dolophine' was rapid and by 32 days was complete for the 5 mg. per kg. dose. Larger doses, however, still produced profound analgesia. Other effects

There was no indication from the usual χ^2 test that the estimates of potency from the various assays differed significantly. The pooled estimate of the relative potency of Intocostrin in terms of Solution Tubocurarine Chloride was 91% with confidence limits of 84 to 98% (P = 0.95). There was no evidence to indicate that the slope for Solution Tubocurarine Chloride differed significantly from the slope for Intocostrin. The estimated slopes varied from 3.78 to 10.04 in the five assays. The weighted average of the slope estimates for the five assays was 6.43.

The assay procedure as described was found to be simple, objective and inexpensive. An assay employing one hundred mice could be performed in approximately half an hour. The mortality among 1,600 mice used in the experiments was less than 6%.

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The animals in Group B were injected subcutaneously. Oddly, with 'Dolophine', subcutaneous administration produced more profound effects than intraperitoneal injections, dose for dose. The first week the dose was 1 mg. per kg. daily, the second week, 1 mg. per kg. t.i.d., the third and fourth weeks, 2 mg. per kg. t.i.d., while for the last 4 weeks, 5 mg. per kg. were injected t.i.d. The drug was given at 8:30 a.m., 12 noon, and 4:30 p.m. Injections distributed equally through the 24-hour day would have been more desirable. During the 8 weeks on the drug, these 6 dogs behaved much the same as those in Group A, although loss of body weight was not so significant until the 5-mg. per kg. dose was started. Careful feeding was necessary thereafter to prevent excessive weight reduction.

A very striking effect in all dogs was marked conditioned salivation, as Collins and Tatum (6) found with morphine. Salivation started immediately after the dogs saw the men who made the injection and before the drug was administered. Conditioned salivation was first noted in the second week of the test. Flow of saliva was very copious and was enhanced by injection of the drug. Salivation may have been an indication of nausea, but vomiting never occurred. It probably was simply another evidence of general parasympathetic stimulation.

None of the animals ever evidenced any desire for the drug such as has been reported in similar studies with morphine in chimpanzees by Spragg (7), and in dogs by Plant and Pierce (8), and Barbour, Hunter, and Richey (9). In fact, the reaction of the dogs was just the opposite, attempts being made to avoid injections or handling.

Constipation occurred during the 8-week injection period. Stools were scanty, hard, and dry as a rule, although occasionally diarrhea developed. The degree of constipation was difficult to estimate owing to irregular and decreased food intake. It was present, however, even during a period of several days when the dogs were fed by stomach tube.

Upon withdrawal of 'Dolophine' at the end of 8 weeks, the results were quite similar to those of Group A. In figure 2 are shown the changes that occurred in heart rate and body temperature. Aside from the fever and rather marked tachycardia, these dogs presented no signs of physical dependence on the drug. Temperature and heart rate fell rapidly in the first 3 days of withdrawal, and then more slowly. After 7 days, temperature and heart rate remained constant, although the heart rate was still higher than the original.

In contrast to our results, Wikler and Frank (10) found that physical dependence to 'Dolophine' readily developed in dogs. However, in man, Isbell and his associates (11) observed only a low level of physical dependence, and Woods, Wyngaarden, and Seevers (12) saw practically no withdrawal signs in monkeys. The reason for these differences is not readily apparent. Variations in the injection intervals and differences in the state of nutrition of the subjects or animals do not appear to be adequate explanations. Further work is indicated to clarify this important point.

2. *Tolerance in rats.* Daily, intraperitoneal injections of 'Dolophine', 5 mg.

of the drug, such as narcosis, sedation, hyenoid posture, and ataxia also were rapidly lost with the standard dose. Curiously, no tolerance whatsoever developed to the cardiac slowing effect either in degree or duration. Van Egmond (5) noted similar results with morphine. Judging by borborygmi, the action of this dose on intestinal motility likewise was not lost. The latter 2 actions are evidences of parasympathetic stimulation, in contrast to the other effects of the drug which are depressant Respiration was too variable to draw any conclusions concerning tolerance.

On cessation of 'Dolophine' administration after 32 days, the animals were observed for withdrawal symptoms. During the 32-day period, the average
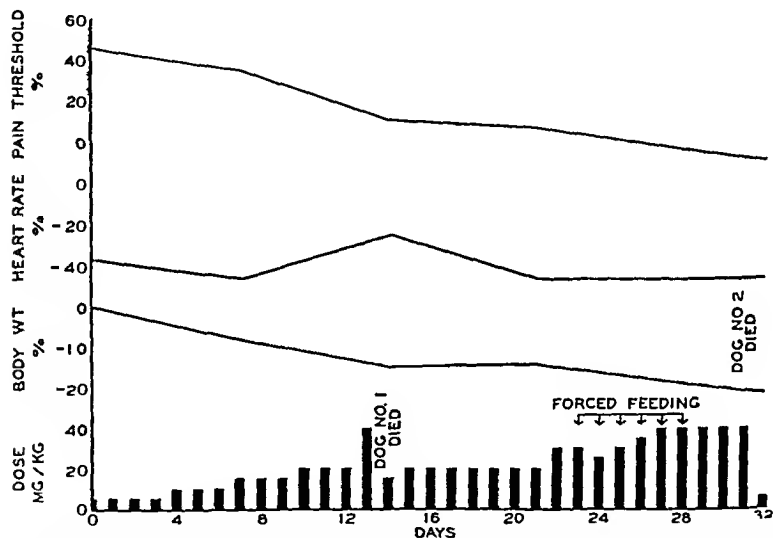

Fig 1 TOLERANCE STUDY OF 'DOLOPHINE' IN 4 DOGS
Although tolerance developed progressively to the analgesic action, the cardiac effect remained unchanged A standard dose of 5 mg of 'Dolophine' per kg was used throughout for tests on pain threshold and heart rate

pulse rate at 8:30 a m (before the first injection of the day) had shown a gradual rise, a probable indication of developing physical dependence On the thirty-second day, the morning pulse rates of the 2 dogs averaged 154 per minute, as compared with their normal of 75 per minute at the start of the tolerance study. After injection of the standard dose of 5 mg per kg , the pulse rates decreased a greater percentage than on the first day. Thereafter, the pulse rate gradually rose and stayed above normal about 24 hours in one dog and 48 hours in the other animal The pulse rate was the only indication of physical dependence, except for a rise of body temperature in one dog amounting to 0 5°F. There was no restlessness, tremors, panting, shivering, or vomiting The dogs were friendly, walked around the room, and seemed to desire to be petted. They appeared to be normal except for malnutrition

The animals in Group B were injected subcutaneously. Oddly, with 'Dolophine', subcutaneous administration produced more profound effects than intraperitoneal injections, dose for dose. The first week the dose was 1 mg. per kg. daily, the second week, 1 mg. per kg. t.i.d., the third and fourth weeks, 2 mg. per kg. t.i.d., while for the last 4 weeks, 5 mg. per kg. were injected t.i.d. The drug was given at 8:30 a.m., 12 noon, and 4:30 p.m. Injections distributed equally through the 24-hour day would have been more desirable. During the 8 weeks on the drug, these 6 dogs behaved much the same as those in Group A, although loss of body weight was not so significant until the 5-mg. per kg. dose was started. Careful feeding was necessary thereafter to prevent excessive weight reduction.

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2. *Tolerance in rats.* Daily, intraperitoneal injections of 'Dolophine', 5 mg.

per kg., were made into young rats for 27 consecutive days. Simultaneously, a similar group was given morphine, 30 mg. per kg. There were 15 animals receiving each drug at the start. Duration of analgesia was determined at weekly intervals, the analgetic effect being tested by the tail-pinching technique of Haffner (13). For this purpose, we used a special forceps described by Kniazuk (14). Results are seen in table 1.

In both groups of animals there was progressive development of tolerance, somewhat more rapid in the 'Dolophine' rats. Tolerance did not become complete to either drug. These doses are probably not equivalent, however, since previous work showed 'Dolophine' to be twice as potent as the opiate. The 'Dolophine' rats grew well, but those on morphine were malnourished. At the
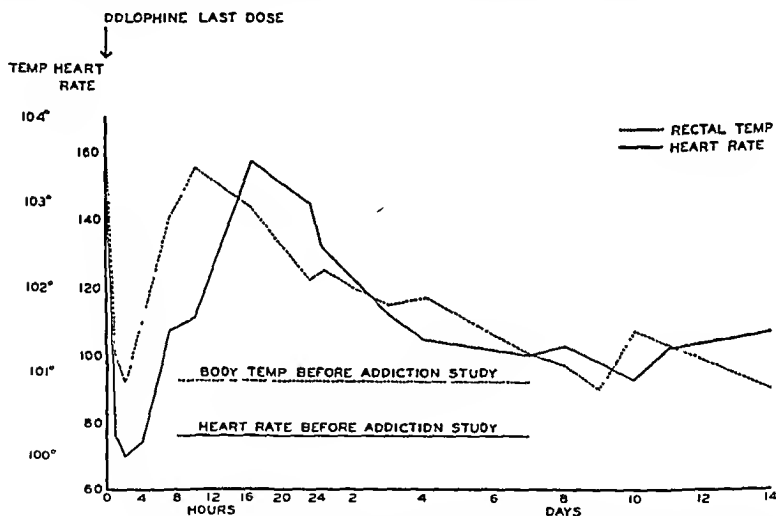

FIG 2 WITHDRAWAL PHENOMENA IN DOGS WHICH RECEIVED INCREASING DOSES OF 'DOLOPHINE' FOR 56 DAYS
There were no other evidences of physical dependence.

end of the test period, an LD_{50} determination was made in the surviving 'Dolophine' group, injections being intraperitoneal. The value was approximately 50 per cent greater than in intolerant rats.

3. *Action on intestinal motility.* In our previous study in dogs, movements were recorded from an exteriorized, skin-covered loop of small bowel. Records were difficult to interpret owing to the fact that the recording balloon was situated outside the intestinal lumen. The present experiments were made in 4 unanesthetized dogs, 2 of which had Thiry's fistulae. In the other 2 animals, a cannula was sutured into the small bowel similar to the technique used by Thomas (15). All 3 parts of the small intestine were studied. For recording, a 3-cm. section of a rubber finger-cot was tied onto the end of a catheter and inserted into the fistula. The catheter was connected to a Harvard membrane blood pressure

manometer with a thin membrane. Exactly 2 cc. of air was introduced into the system. This procedure was designed to minimize mechanical stimulation of the gut.

A total of 29 experiments were run. In 3 of these, morphine sulfate was used, while demerol was tested 10 times. The remainder of the records were made with 'Dolophine'. The doses used were 0.5 to 1.0, 1.0, and 5.0 mg. per kg., respectively, for 'Dolophine', morphine, and demerol. Injections were made slowly into a leg vein. In 9 experiments, physostigmine salicylate, 0.25 mg. per kg., was injected subcutaneously about 15 minutes beforehand to increase bowel motility. In 13 experiments, when the action of the test drug had developed,

TABLE 1

*Comparison of the development of tolerance to the analgesic action of
Morphine and 'Dolophine' in rats*

Doses of the former were 30 mg. per kg. and for the latter 5 mg. per kg. The drugs were administered once daily by intraperitoneal injection.

DRUG		DAYS ON DRUG				
		1	7	15	22	27
'Dolophine'	Average Duration of Analgesia (min.)	116	69	52	30	31
	Number of Rats	15	10	10	10	10
	Average Weight (grams)	79.2	105.3	130.0	163.6	179.7
	LD_{50} mg./kg. i.p.	24				37
Morphine Sulfate	Average Duration of Analgesia (min.)	145	105	86	81	47
	Number of Rats	15	13	13	13	7
	Average Weight (grams)	76.7	94.8	108.3	115.4	116 3

atropine sulfate, usually 0.65 mg. intravenously, was given. Results in the series were absolutely consistent. 'Dolophine', demerol, or morphine always stimulated the small bowel. Stimulation involved tone, amplitude, and rate of segmental contractions, or peristalsis. Sometimes all 3 were increased. When the motility was enhanced by physostigmine, further stimulation followed administration of 'Dolophine' or demerol. On the other hand, atropine always inhibited motility, sometimes completely. In figure 3 are shown some of the records.

The results with demerol came as a surprise because of many reports in the literature about its atropine-like action. The present findings show distinctly the opposite, namely, parasympathomimetic effects. A similar result on the heart will be discussed later.

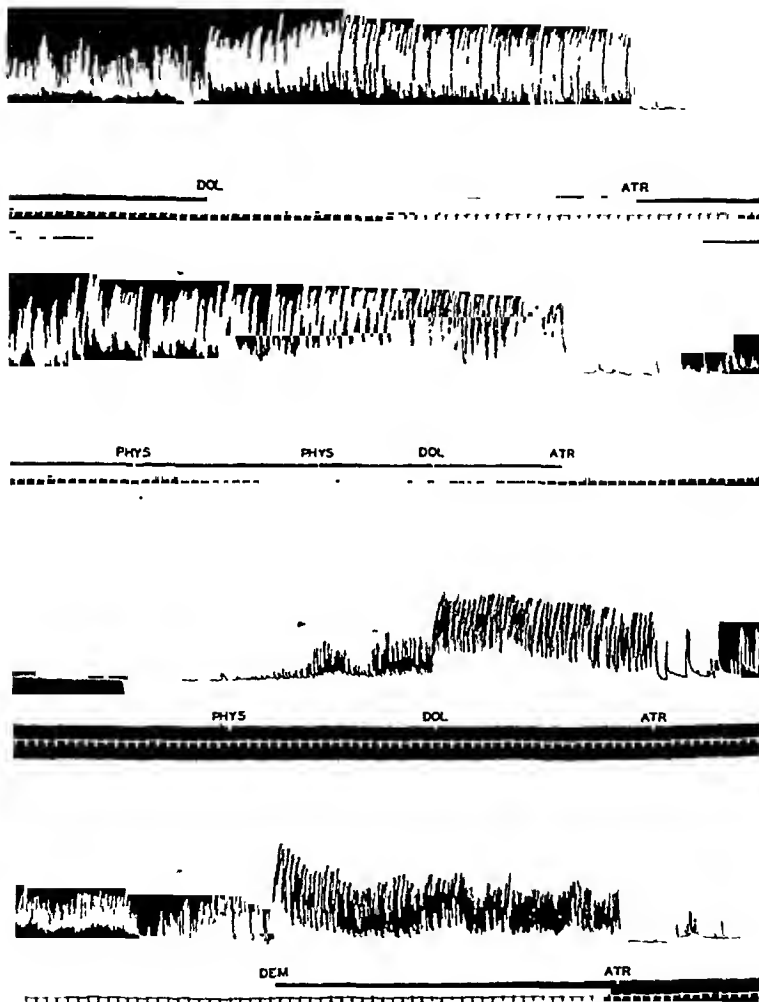Tests for constipating action were carried out in rabbits by the method of Sato

FIG 3   STUDIES OF INTESTINAL MOTILITY IN UNANESTHETIZED DOGS
From above down, the first three records are of jejunal activity, while the last was taken
from the ileum   Symbols represnt the following   DOL—'Dolophine', 2 mg per kg. i.v.,
PHYS—physostigmine salicylate, 0 25 mg per kg s c , ATR—atropine sulfate, 0 65 mg
i v., and DEM—demerol, 5 mg per kg i v   Time in minutes

(16).[2]   For comparison both 'Dolophine' and morphine were run   Results are
found in table 2   Apparently, 'Dolophine', 2 mg per kg., produced a consti-

[2] These tests were kindly performed by Mr C L Rose.

pating effect equal to that of morphine sulfate, 5 mg per kg   This ratio is approximately the same as the analgesic potency of the 2 substances   With both drugs, the mechanism was probably a prolonged increase of tone or spasm.

4 *Quantitative measurements of respiratory changes*   Using an automatic recording double spirometer system (17), continuous measurement of the volume

TABLE 2

The constipating action of 'Dolophine' and Morphine in rabbits   The drugs were injected intravenously

DRUG	DOSE	NUMBER OF RABBITS	TOTAL NUMBER OF SCYBALA PASSES IN 8 HOURS
	mg/kg		
'Dolophine'	1	12	76
	2	7	24
Morphine	2	5	121
Sulfate	5	5	14
Controls	--	24	112

TABLE 3

Comparison of effectiveness of various stimulants against respiratory depression by 'Dolophine'

STIMULANT DRUG	DOSE mg/kg I V	NUMBER OF DOGS	MEAN VOL EXPIRED AIR (LITERS/MIN FOR 30 MIN)		STIMULATION
			Following 'Dolophine'	After Stimulant	
					per cent
d Desoxyephedrine Sulfate	2	3	0 802	1 383	72 4
d Benzedrine Sulfate	2	3	1 090	1 650	51 4
Ephedrine HCl	5	2	0 891	1 239	39 0
	2	1	1 183	1 233	4 2
l *n* Propyl Theobromine	10	2	1 169	1 408	20 4
Nikethamide	30	2	1 007	1 149	14 1

of expired air was made in dogs anesthetized with sodium phenobarbital.   When respiration became stabilized, a control period of 30 minutes was recorded 'Dolophine', 2 mg per kg , was then injected intravenously and the respiratory minute volume measured for exactly one-half hour   Approximately a 50 per cent reduction of respiration followed this dose   At the end of the half hour, a respiratory stimulating drug was injected intravenously   The per cent increase of expired air volume was calculated by comparing the minute volumes for the half hour periods before and after the stimulant   Results are given in table 3.

The sympathomimetic compounds produced the greatest respiratory stimulation. Of these, *d*-desoxyephedrine was the most potent Nikethamide and 1-*n*-propyl theobromine caused only a moderate increase. The latter compound was the most potent respiratory stimulant of a series of xanthine derivatives previously tested in these laboratories (18).

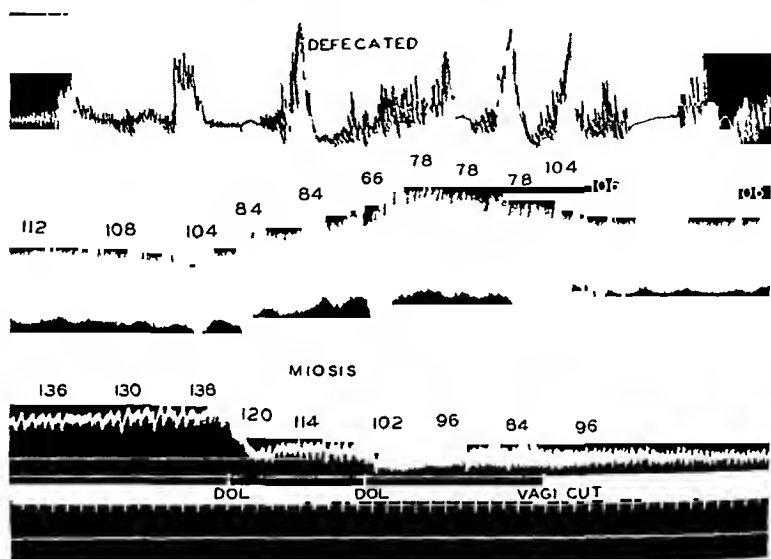5 *Cross-circulation experiments* Using the technique of Heymans and Heymans (19), cross-circulation studies were made to determine whether parasym-

FIG 4 CROSS CIRCULATION EXPERIMENT IN ANÆSTHETIZED DOGS
(HEYMAN'S TECHNIQUE)
Recipient's head and body were connected only by the vagus nerves From above down are duodenal movements and blood pressure of recipient's body, while the lower tracing is the donor's blood pressure record Numbers represent heart rates The baselines for blood pressure of the recipient and donor are the top and bottom lines, respectively At DOL, 'Dolophine', 2 mg per kg , was injected into recipient's carotid artery The cardiac slowing and increased intestinal motility after 'Dolophine' prove the stimulation to be of central origin Time in minutes

pathetic stimulation by 'Dolophine' was of central origin Under barbiturate anesthesia, the head of a dog was transfused with blood from a donor dog The femoral blood vessels of the latter were connected to the common carotid arteries and external jugular veins of the recipient All other vessels and structures in the neck of the recipient were ligated or crushed except the vagus nerves Thus the latter were the only connection between the head of the recipient and its body That the spinal cord was crushed was verified by necropsy at the conclusion of the experiment Blood pressure and intestinal motility of the recipient's body were then recorded in the usual manner The blood pressure of the donor dog was also registered and both animals heparinized

Upon injection of 'Dolophine' into the tubing carrying arterial blood to the recipient's head, the heart rate of this animal's body was slowed and the intestinal movements augmented. Figure 4 is a record of one of the experiments. This proved the central stimulating action of 'Dolophine'. Further evidence was afforded when the vagus nerves were severed, the bradycardia being immediately abolished. Intestinal movements decreased somewhat on vagal section, but did not return entirely to the original before 'Dolophine'. It is possible that the drug may also act as a stimulant to the parasympathetic nerve endings in the gut, since injection of the drug intravenously into the recipient's body sometimes produced some stimulation. Morphine is known to have a local spasmogenic effect on the small bowel (Plant and Miller, 20) after extrinsic nerve denervation. An explanation of this action may be the effect of morphine on cholinesterase, since Bernheim and Bernheim (21) have shown that the opium alkaloid depresses this enzyme. 'Dolophine' possibly may possess this latter action.

6. *Parasympathomimetic action of demerol on the heart.* Since our work showed that demerol exerted a spasmogenic effect on the bowel, further studies were done to determine if this drug resembled morphine and 'Dolophine' in action on heart rate. Tests were made on 12 dogs trained to lie quietly on a table. These animals showed no appreciable spontaneous change of cardiac rate over a period of hours. Demerol, 10 mg. per kg., was injected intraperitoneally into 7 dogs while the other 5 animals received a dose of 20 mg. per kg. The mean pulse rate fell from 69 to 58 per minute in the first group, while the dogs given the larger dose responded with a drop in cardiac rate from 72 to 48 per minute. These values, while not quite so large as those obtained in similar experiments with 'Dolophine', represent very definite cardiac slowing. In 2 of the animals given the 20-mg. dose, tachycardia immediately followed intravenous injection of 0.65 mg. of atropine sulfate. Electrocardiograms taken during the course of these experiments showed only a simple sinus bradycardia. These results are identical with those obtained with 'Dolophine' (1).

<div align="center">SUMMARY</div>

1. In dogs receiving injections daily for as long as 56 days, tolerance developed to the analgesic and narcotic actions of 'Dolophine', when the dose was gradually increased and the intervals of administration were shortened. No tolerance to cardiac slowing and intestinal motility effects occurred.

2. The only withdrawal phenomena noted in these dogs were tachycardia and low-grade fever.

3. Rats developed partial tolerance to the analgesic action of 'Dolophine' when given a constant single daily dose for 27 days.

4. 'Dolophine' resembled morphine in its action on the intestine. It increased the motility of the small bowel of unanesthetized dogs and produced constipation in rabbits and dogs.

5. Demerol stimulated the movements of the small intestine and slowed the heart rate. These results are directly opposed to those of investigators who claim an atropine-like effect for the drug.

6. Respiratory depression following 'Dolophine' was adequately counteracted by d-desoxyephedrine, d-benzedrine, or ephedrine, the first-named drug being the most potent. Nikethamide and 1-n-propyl theobromine were less efficient.

7. By cross-circulation experiments the parasympathomimetic effects of 'Dolophine' on heart rate and intestinal motility were shown to be of central origin.

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THE TOXICITY AND GENERAL PHARMACOLOGY OF N_1-p-CHLORO-PHENYL-N_5-ISOPROPYL BIGUANIDE

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The introduction of N_1-p-chlorophenyl-N_5-isopropyl biguanide, also known as 4888 and paludrine, is distinctly a British contribution of her war effort to medicine for the treatment of malaria (1, 2, 3). The results of the antimalarial tests in birds and clinical studies of American investigators have been summarized by Wiselogle (4), and more recent pharmacologic data have been presented by Schmidt and his associates (5, 6). We would like to add our observations on the general pharmacology and toxicity of the same product. Our material came from two sources: E. I. du Pont de Nemours & Company, Wilmington, Delaware, and Imperial Chemical Industries, Limited, Manchester, England. Both specimens were in form of the hydrochloride. They softened at 228° to 229°C. and melted at 241° to 243°C. There was no depression of the mixed melting point. Both salts are bitter to taste, and soluble in water to the extent of 1%. It may be stated that the two samples are indistinguishable from each other.

1. Acute Toxicity. By intravenous or oral administration of N_1-p-chlorophenyl-N_5-isopropyl biguanide hydrochloride to mice, rats, guinea pigs, and rabbits, median lethal doses ± standard errors (LD_{50} ± S. E.) were determined. For comparative purposes, similar tests were made with quinine dihydrochloride and quinacrine hydrochloride, and their LD_{50}'s computed from the crude data.

The results are shown in table 1. It will be noted that in all animals studied N_1-p-chlorophenyl-N_5-isopropyl biguanide HCl, either intravenously or orally, is more toxic than quinine 2HCl. The new preparation given intravenously is also more toxic than quinacrine HCl to mice; but in larger animals, such as rats, guinea pigs, and rabbits, quinacrine HCl is more toxic by the intravenous route. Orally, however, it remains less toxic than the biguanide. It may be also pointed out that in mice and rats the intravenous and oral LD_{50}'s of the new preparation are close to each other, whereas those of quinine 2HCl and quinacrine HCl are far apart, the oral being several times greater than the intravenous.

In our experiments, the toxic manifestations in mice and rats following oral administration of lethal or sublethal doses of the biguanide were the same as described by Schmidt and co-workers (5). When given by vein, initial tonic convulsions were observed, followed by prostration prior to death in mice, rats, and guinea pigs. Rabbits receiving lethal doses of the drug became prostrated for prolonged periods of time before they died.

The oral toxicity of the biguanide in rats, as recorded by us, was definitely higher than that reported by Schmidt and his associates (5). We repeated the test several times, and each time found the LD_{50} well within 100 mg. per kg. Our rats, unquestionably healthy, were purchased from a dealer who was not certain

of the strain, while the Cincinnati group used the Sprague-Dawley stock. It remains to be seen whether the discrepancy of results between Schmidt's labo-

TABLE 1

Comparison of acute toxicity of N_1-p-Chlorophenyl-N_5-isopropyl Biguanide HCl, Quinine 2HCl, and Quinacrine HCl

ANIMAL	ROUTE OF ADMINISTRATION	BIGUANIDE HCl		QUININE 2HCl		QUINACRINE HCl	
		No. of Animals Used	LD$_{50}$ ± S. E	No. of Animals Used	LD$_{50}$ ± S E	No. of Animals Used	LD$_{50}$ ± S. E
			mg. per kg.		*mg. per kg.*		*mg per kg*
Mouse	Intravenous	60	23.03± 1.10	25	95.79 ± 4.67	30	38.0 ± 0.9
	Oral	20	27.49 ± 1.76	40	660.4 ± 40.9	20	556.8 ± 30.6
Rat	Intravenous	40	33.10 ± 1.16	20	78.2 ± 6.65	30	29.4 ± 4.9
	Oral	40	58.20 ± 3.50	24	1392 ± 192	48	659.8 ± 30.4
Guinea pig	Intravenous	15	39.51 ± 2.41	17	57.07 ± 3.99	18	14.19 ± 1.09
Rabbit	Intravenous	14	44.85 ± 2.78	18	34.77 ± 1.98	17	8.89 ± 0.28
	Oral	21	243 ± 21	20	641 ± 56	17	433 ± 54

TABLE 2

Repeated administration of N_1-p-Chlorophenyl-N_5-isopropyl Biguanide HCl in rats by mouth

FIXED DOSE PER RAT	DOSE RANGE BASED ON INITIAL WEIGHT	NUMBER OF RATS IN GROUP	RESULTS OF REPEATED ADMINISTRATION
mg.	*mg. per kg.*		
0.5	5.62–7.14	5	All 5 rats survived 20 doses
1	11.36–14.92	5	Ditto
2	23.26–27.77	5	Ditto
3	35.71–42 25	5	Ditto
4	52.63–64.52	5	1 died after 5th dose; 2 after 9th; and 2 survived 20 doses
5	53.76–60.98	5	3 died after 1st dose; 1 after 2d; and 1 survived 20 doses
6	75.00–95.24	5	1 died after 1st dose; 4 after 3d
8	88.88–140.35	10	8 died after 1st dose; 2 after 2d
10	123.46–178.57	5	3 died after 1st dose; 2 after 2d

ratory and ours is due to the difference in strain of rats, or some other reason. The existence of strain-difference of response among rats to thiourea has been reported by Dieke and Richter (8).

2. Chronic Toxicity. Nine groups of rats, as shown in table 2, were each

given by stomach tube the new antimalarial in 0.5 and 1% solutions, depending on the size of the dose. Mixing the drug with food was avoided because of the bitterness of the substance. Medication was repeated each day of the week except Saturdays and Sundays, and a fixed dose was used for each group. A control group without medication was also run. The animals weighed between 56 and 93 grams, average 75.5 grams. The dose ranges based on initial body weight are listed in the second column of table 2. The entire experiment lasted 4 weeks.

By inspection of table 2, it is apparent that young rats tolerated daily doses of 6 up to 42 mg. per kg. for 4 weeks. Their average body weight at the end of the experiment was indistinguishable from that of the control group (not shown in the table). The next 2 groups can be considered together since the amounts of the drug per kg. administered fell close together. Three died after the first dose; 1 after the second; 1 after the fifth; and 2 after the ninth. Three survived 20 doses. The last 3 groups all died shortly after the start of the experiment: the majority succumbed to the first dose, and the rest failed to tolerate 2 or 3 doses.

All our rats, dead or upon sacrifice, were subjected to necropsy by Dr. Paul N. Harris. There were no pathological changes which could be detected grossly or microscopically. This confirms the findings of Schmidt, Hughes, and Smith (5). The cause of death in our experimental animals by repeated administration must have been due to acute systemic effects of the biguanide.

3. Circulation and Respiration. In cats anesthetized by ether or barbiturates, or pithed, the new antimalarial drug injected intravenously caused a uniform fall of carotid blood pressure with prompt recovery. The effective dose ranged from 1 to 16 mg. Although no attempt was made to determine the cause of the fall, it was probable that cardiac depression was at least partly, if not entirely, responsible. This was illustrated in frogs' hearts perfused according to the method of Howell and Cooke (7). Concentrations of 1:20,000 to 1:10,000 reduced both the amplitude and rate of the ventricle.

During the fall of blood pressure in cats, the respiratory rate was accelerated, but its depth diminished—returning to the original level with the blood pressure. The respiratory change could, therefore, be a reflex response to depressor action.

4. Isolated Smooth Muscle Organs. The biguanide in concentrations of 1:50,000 to 1:12,500 relaxed isolated rabbits' small intestines suspended in Locke-Ringer's solution. A dilution of 1:200,000 had no effect on isolated guinea pigs' intestines, but inhibited histamine spasm, although it was far less effective than an antihistamine agent such as β-dimethylamino ethyl benzhydryl ether HCl.

Strips of isolated guinea pigs' uteri immersed in Locke's solution contracted with the biguanide in solutions stronger than 1:40,000. A dilution of 1:100,000 had no effect on the isolated rabbit's uterus, but brought about relaxation during the contraction caused by epinephrine.

5. Blood Sugar. Since some guanidine derivatives lower blood sugar (9), it was decided to investigate the new antimalarial for the same effect in 4 rabbits. The drug was injected by the marginal ear vein in the dose of from 20 to 40 mg.

per kg., and the blood sugar determined at hourly intervals by the method of Hagerdorn and Jensen (10).

The results appear to indicate that the drug caused a slight hypoglycemia in all 4 animals. The drop in blood sugar per 100 cc., however, was not remarkable: in 1 rabbit receiving 20 mg. per kg., it fell from 101 to 95 at the end of the fourth hour; in 2 on 30 mg. per kg., from 106 and 108 to 99 and 93, respectively, at the end of the fifth hour; and in another on 40 mg. per kg., from 86 to 52 at the end of the fourth hour.

SUMMARY

1. The acute toxicity of N_1-p-chlorophenyl-N_5-isopropyl biguanide HCl has been compared with quinine 2HCl and quinacrine HCl in mice, rats, guinea pigs, and rabbits.

2. The biguanide has been repeatedly administered in various doses to rats. No pathologic lesions can be demonstrated, and death from large doses may be attributed to acute systemic effects.

3. The biguanide lowers blood pressure with an acceleration of respiratory rate in anesthetized cats.

4. With the exception of the isolated guinea pig's uterus, which responds by contraction, the biguanide has an inherently relaxing property on the isolated rabbit's uterus and intestine, and the isolated guinea pig's intestine.

5. The action of the biguanide on the blood sugar of rabbits is suggestive of a slight hypoglycemia.

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EVALUATION OF CHEMOPROPHYLACTIC ACTIVITY BY THE SUB-CUTANEOUS IMPLANTATION OF PELLETS OF SULFONAMIDES IN MICE[1]

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In the evaluation of chemoprophylactic or chemotherapeutic activity of the sulfonamides, the maintenance of a known and essentially constant sulfonamide concentration in the blood is desirable. For this to be accomplished in mice, the oral or parenteral administration of sulfonamides must be repeated frequently throughout the day and night because of the rapid excretion of these compounds, a reflection of the high metabolic rate of this animal species. When experiments are designed to continue throughout a period of several days, such a procedure becomes laborious and time-consuming and not infrequently causes death due to traumatic injury. The method employed by Bieter, et al. (1) and used extensively by others, particularly by Litchfield, White and Marshall (2), in which the sulfonamide is incorporated in the diet, possesses certain disadvantages: 1) variations in the feeding habits of the mice cause the concentration of drug in the blood to fluctuate; 2) handling and bleeding the mice disturb the regularity of their feeding habits; and 3) mice infected with certain species of microorganisms exhibit loss of appetite, a circumstance that leads to a sharp decrease in, and very often a complete cessation of, the consumption of the drug-diet. As a result, the concentration of sulfonamide in the blood decreases and protection against the development of a lethal infection soon is incomplete or absent. In view of these considerations, a simple method of establishing and maintaining an essentially constant concentration of sulfonamide in the blood of mice, regardless of food consumption, and without involving laborious technics, is most desirable.

Uniformly compressed, hard pellets of sulfonamides were implanted subcutaneously in mice. After an "adjustment period," during which the concentration of drug in the blood rose to a high level and then decreased, a "plateau period" eventually was established; this period was characterized by a concentration of drug in the blood that decreased gradually during a period of many days or even weeks. Throughout this second period conditions were particularly advantageous for testing the resistance of the animals to infections caused by the injection of pathogenic microorganisms. In this study, the method was used to

compare the chemoprophylactic activities of sulfamerazine and sulfadiazine in experimental pneumococcal and streptococcal infections in mice.

METHODS. Compressed pellets (25 mgm) of sulfamerazine and of sulfadiazine were prepared in the following manner.[4] The drug (26 25 grams) was mixed with 5 per cent 'Methocel'[5] (12.5 grams); the mixture was triturated thoroughly, was dried at room temperature, and then was forced through a #20 stainless steel wire screen. The resulting mixture of granules and powder was separated by sifting through 86-mesh bolting cloth and the powder again was granulated with 'Methocel' and was reduced to 20-granule size. The two granular portions were mixed thoroughly and three drops of mineral oil were added to serve as a lubricating agent (this is necessary for the compression of the pellets). Finally, 3.4 grams of boric acid (sifted through a 90-mesh bolting cloth) were incorporated to prevent binding during compression of the pellets. The granules were passed through a tablet machine using a $\frac{5}{32}$ inch punch and die. The resulting pellets weighed approximately 29 mgm and contained 25 0 ± 0.6 mgm. of sulfonamide.

Healthy mice of 18 to 25 grams weight and of the same strain were used in all experiments. Each animal was narcotized by the intraperitoneal administration of pentobarbital. The hair covering the abdomen was removed with clippers and a small incision was made in the skin. A pellet of sulfonamide was inserted subcutaneously over the flank as far as possible from the incision, which then was closed with a single stitch. Aseptic technic was not employed and no evidence of infection was seen in any of the 1132 mice implanted in the manner described; healing of the incision essentially was complete within two days. When more than one pellet was implanted, both flanks were used and each pellet was placed in a separate position. Using this method, the position of the pellets became fixed within a few hours and "wandering" rarely was seen. The pellets remained intact for long periods and usually could be palpated during the course of at least one month following implantation.

Since too frequent removal of blood deleteriously affects the health of mice, each group of mice was divided into two sub-groups that were bled alternately. Determinations of the concentration of free sulfonamide in blood (0.05 cc.) drawn from the tail were made, according to the method of Bratton and Marshall (3, 4).

RESULTS. The concentration of sulfonamide in the blood, produced during the course of a 20-day period by the implantation in mice of various numbers of pellets of sulfamerazine or of sulfadiazine, is shown in figure 1. Following implantation, the concentration in the blood rose rapidly to a peak, usually within 24 hours, and then decreased fairly rapidly during several days. After about 6 days the "plateau period" was reached, during which the concentration of drug in the blood decreased very gradually from day to day. Depending upon the number of pellets originally implanted, a period of from 6 to 12 weeks was required for the disappearance of sulfonamide from the blood. If sulfonamide-implanted mice were infected about one week after implantation, the change in concentration of drug in the blood was quite insignificant during the critical period (2 to 4 days) following infection. In 15 experiments, the average concentration of sulfonamide in the blood on the fourteenth day was 83 per cent of that on the seventh day. Further evidence of the value of the implantation method was afforded by the observation that the food consumption and general health of the mice did not appear to be affected by the procedure.

The relation of dosage to blood level, following the subcutaneous implantation of sulfamerazine and sulfadiazine, confirmed results previously obtained by other routes of administration and in other animal species (5, 6). It was found that implantation with twice as many pellets of sulfadiazine as of sulfamerazine was required to permit the maintenance of closely similar concentrations in the blood.

In order to determine the role of excretion in the maintenance of these relationships, each of a group of 10 mice was implanted with two pellets of sulfamerazine, while four pellets of sulfadiazine were placed in each animal of a similar group. In the blood of the two groups, the concentration of free sul-
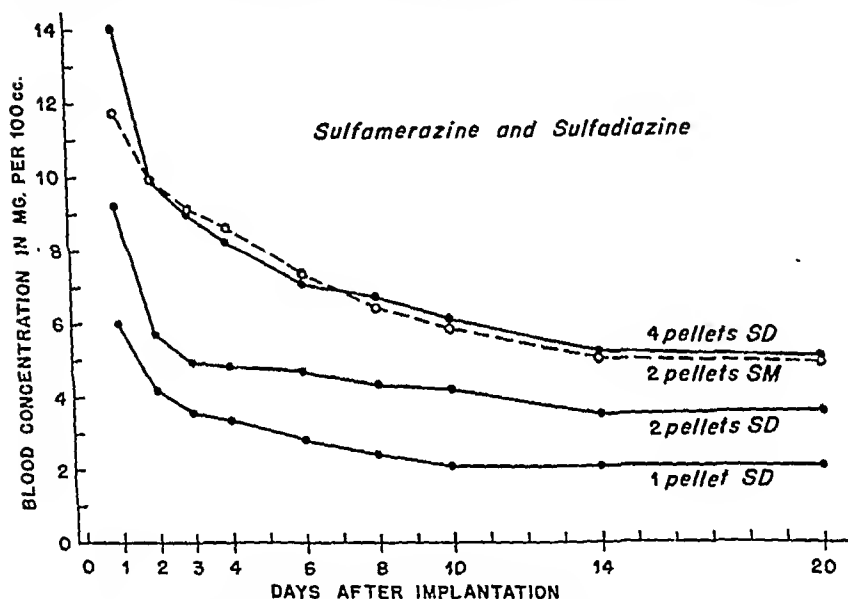

FIG. 1. CONCENTRATION OF "FREE" SULFONAMIDE IN THE BLOOD OF MICE SUBCUTANEOUSLY IMPLANTED WITH TWO 25 MGM. PELLETS OF SULFAMERAZINE OR VARYING NUMBERS OF 25 MGM. PELLETS OF SULFADIAZINE

fonamide was practically identical. The urine of the group was collected during each 24-hour period for four days and the content of free and of total sulfonamides was determined; the results are presented in table 1.

Since a greater amount of sulfadiazine than of sulfamerazine was excreted while the concentrations in the blood were equal, it is apparent that sulfadiazine, under the conditions of these experiments, as under other experimental conditions (5, 6, 7), is excreted by the kidney at a more rapid rate than is sulfamerazine. In agreement with earlier work, it is evident also that the difference in the rate of renal excretion is more marked with the free forms of these two drugs than with the conjugated forms.

Unfortunately some sulfonamides are unsuitable for comparative studies by

the technic of implantation. The rate of absorption of the drugs by the subcutaneous tissues and the rate of their excretion must be such as to permit the maintenance of closely similar "plateau periods." Thus, the implantation of pellets of sulfathiazole in mice did not produce adequate concentrations in the blood, as may be seen in figure 2. Within two days after implantation, sulfathia

TABLE 1

Urinary excretion of sulfonamides by mice implanted subcutaneously with sulfadiazine (4 pellets = 100 mgm.) or sulfamerazine (2 pellets = 50 mgm.)

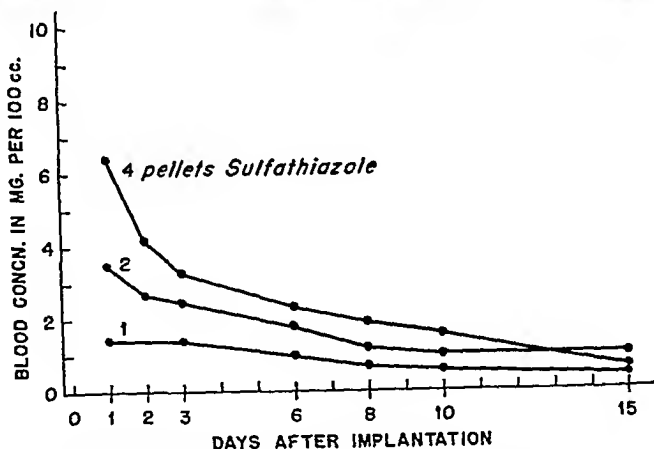| TIME INTERVAL	SULFADIAZINE (4 PELLETS)			SULFAMERAZINE (2 PELLETS)		
	Free	Acet.	Total	Free	Acet	Total
days	*mgm.*	*mgm*	*mgm*	*mgm.*	*mgm.*	*mgm*
0–1	2.91	1.70	4.61	2.17	1.45	3.62
1–2	3.02	2.18	5.20	1.79	1.98	3.77
2–3	2.76	1.08	3.84	1.42	0.86	2.28
3–4	2.34	1.24	3.58	1.64	1.01	2.65
0–4	11 03	6 20	17 23	7.02	5.30	12 32

FIG. 2. CONCENTRATION OF "FREE" SULFONAMIDE IN THE BLOOD OF MICE SUBCUTANEOUSLY IMPLANTED WITH VARYING NUMBERS OF 25 MGM PELLETS OF SULFATHIAZOLE

zole pellets became soft and pliable, and within ten days these had disintegrated completely. It is reasonable to predict, however, that the more slowly excreted sulfonamides, e.g., sulfapyridine and sulfapyrazine, may be studied in this manner.

CHEMOPROPHYLACTIC EVALUATION. In order to evaluate sulfonamides chemoprophylactically in mice, mortality due to infection should be correlated with the

concentration of free sulfonamide maintained in tissue fluids during infection. Although the concentration in the plasma water is thought by some to have the greatest significance, these studies, due to the severe restriction on the amount of blood available, made use of whole blood.   By varying both the number of pellets implanted and the time-interval between implantation and infection, any reasonable concentration of sulfamerazine and sulfadiazine in the blood could be obtained.   At least one week elapsed after implantation before the animals were infected in order to permit the concentration of drug to enter the "plateau period."   On the day of infection, blood (0.05 cc.) was drawn from the tail vein of each mouse for the determination of the concentration of free sulfonamide present at the time of infection.

  1. *Streptococcus hemolyticus.*   A total of 442 mice was used in the determination of the comparative chemoprophylactic activity of sulfamerazine and sulfadiazine in experimental infections with a virulent strain (#1685) of *Streptococcus hemolyticus.*   All mice were injected intraperitoneally with approximately 1000 M.L.D. of a suspension of the organisms and were observed for a period of one week.   It was not found necessary to extend this period of observation, since deaths from infection invariably occurred within this period or not at all.   The $S.B.C._{50}$[6] for free sulfamerazine was 2.4 mgm. per 100 cc., while that for free sulfadiazine was 0.8 mgm. per 100 cc.   It must be emphasized, however, that the minimal concentration of sulfonamide in the blood that was required to protect *100 per cent* of the infected animals was the same for the two drugs (figure 3).

  2. *Pneumococcus.*   When mice implanted with sulfamerazine or sulfadiazine were injected intraperitoneally with 10 M.L.D. of a virulent strain of *D. pneumoniae* (Type I), a relatively high concentration of free sulfonamide in the blood was required to protect the animals against the development of a fatal infection during the subsequent 14-day period.   The $S.B.C._{50}$ of sulfamerazine was 14.8 mgm. per 100 cc. and that for sulfadiazine was 16.4 mgm. per 100 cc., a difference that is not considered to be significant (figure 4).   Since the values for $S.B.C._{50}$ and the slopes of the survival curves for the two drugs do not differ significantly, it may be concluded that, under the experimental conditions employed, sulfamerazine and sulfadiazine have almost identical protective action in (Type I) pneumococcal infections.

  DISCUSSION.   The well-known fluctuations in the concentration of drug in the blood that result from the administration of sulfonamides by various practical methods such as gastric intubation, parenteral injection, or admixture with the diet, are avoided, at least in the case of sulfadiazine or of sulfamerazine, by the implantation of hard pellets of the drug under the skin.   This technic of administration leads to the prompt production of a relatively high blood-concentration, which falls rapidly during the following few days until a "plateau period" is reached.   During this period, which lasts for four weeks or more (depending on the number of pellets implanted), the concentration falls very gradually without

  [6] The concentration of drug in the blood which is required to permit the survival of 50 per cent of a group of animals.
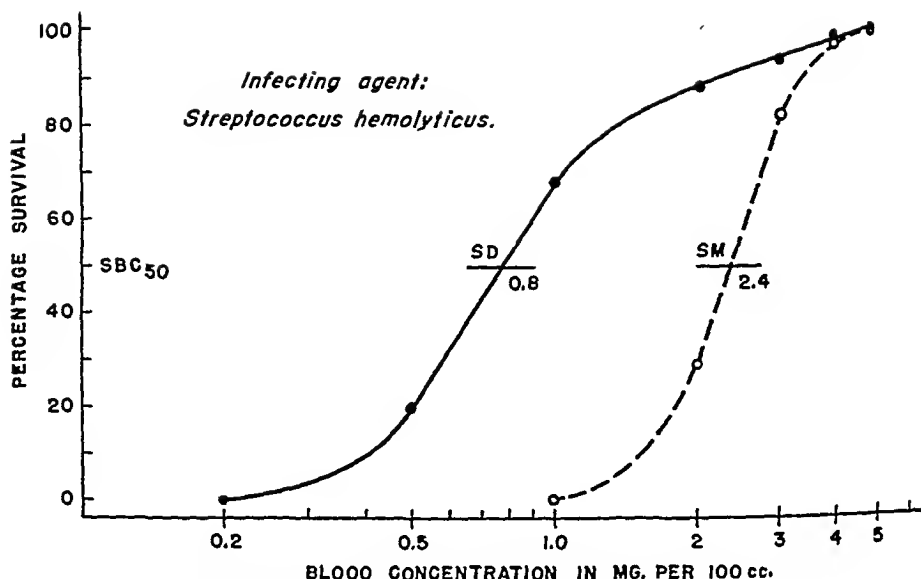
Fig. 3. Percentage Survival of Mice Subcutaneously Implanted with Pellets of Sulfadiazine or of Sulfamerazine, Following Infection with Streptococcus hemolyticus (#1685)

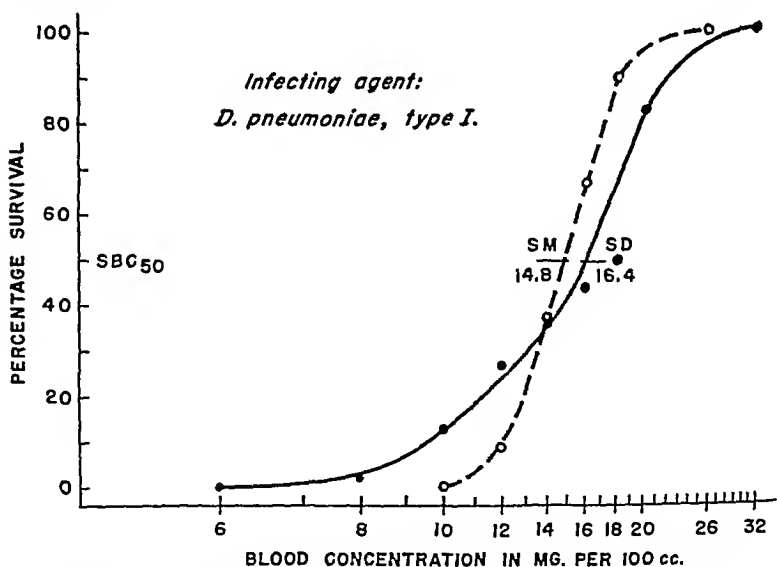The blood concentration of each drug required to protect 50 per cent of the animals (SBC_{50}) is indicated.

Fig. 4. Percentage Survival of Mice Subcutaneously Implanted with Pellets of Sulfadiazine or of Sulfamerazine, Following Infection with D pneumoniae (Type I)

The blood concentration of each drug required to protect 50 per cent of the animals (SBC_{50}) is indicated.

detectable fluctuations. The height of the "plateau" concentration of drug can be controlled by varying the number of pellets of sulfonamide initially implanted. Since mice with various maintained concentrations of sulfonamide in the tissue fluids are thus provided, it is possible to determine the chemoprophylactic activity of a drug by comparing the incidence of survival among a group of infected animals, with the concentration of drug in the blood, blood plasma, or plasma water. In these preliminary studies attention was given only to the concentrations produced in whole blood.

The observation that protection of 50 per cent of a group of mice against a lethal infection with streptococci was afforded by a concentration of 0.8 mgm. of sulfadiazine per 100 cc. of blood, while 2.4 mgm. of sulfamerazine was required for the same protection, suggested the possibility that a proportion of the sulfamerazine in the blood of the mice was chemoprophylactically inactive. Experiments designed to disclose the mechanism of this inactivation appeared to indicate that protein-binding was not responsible for this apparent interference; further work will be necessary to account for the apparent difference between the two compounds. The therapeutically important fact should be pointed out, however, that the concentration of sulfamerazine in the blood required to protect approximately 100 per cent of mice against a lethal infection with streptococci was quite indistinguishable from that required with sulfadiazine.

It is interesting to note also that the same concentrations of the two drugs are required for the chemoprophylaxis of (Type I) pneumococcal infection.

SUMMARY

A method of implanting pellets of sulfonamide in mice is described by means of which a very gradually decreasing concentration of drug is maintained in the blood. This method, particularly useful with the more slowly excreted sulfonamides, affords a simple means for evaluating the activity of a sulfonamide in protecting against various bacterial infections. A comparative study of sulfamerazine and sulfadiazine indicated that for equivalent concentrations of drug in the blood to be maintained, twice as many pellets of sulfadiazine as of its mono-methyl derivative, sulfamerazine, must be implanted. The concentration of free drug found necessary for protection of 50 per cent of mice against lethal hemolytic streptococcal infection, was for sulfadiazine, 0.8, and for sulfamerazine, 2.4 mgm. per 100 cc. of blood; this difference between the two drugs was seen only at critically low blood levels, since the concentrations required to protect 100 per cent of mice did not differ. Against lethal pneumococcal (Type I) infection, the concentration for protection of 50 per cent of mice was for sulfadiazine, 16.4, and for sulfamerazine, 14.8 mgm. per 100 cc. of blood.

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THE PHARMACOLOGY OF A NEW SERIES OF CHOLINE SALTS

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Perchloric acid yields with many choline compounds the corresponding perchlorate usually as a non-hygroscopic stable salt, which may be readily isolated and purified by recrystallization. A series of compounds possessing pharmacologic interest has been prepared by this process. The present investigation was conducted to compare these compounds with the salts of choline and acetyl choline that are in general therapeutic use and in addition, because of the ease of isolation as perchlorates, certain new derivatives have been studied. Previously the pharmacology of the nitrate ester of choline perchlorate was reported (1).

Physical and chemical properties of compounds studied. We have prepared the perchlorate salt of each of the following choline esters: acetyl, benzoyl, furoyl, thenoyl, nicotinyl and acetyl-β-methyl. In addition, choline perchlorate was prepared. These compounds are stable, non-hygroscopic crystalline salts having a sharp melting point and were identified by carbon and hydrogen analysis. Their chemistry has been reported elsewhere (2).

Depressor response. In 10 dogs (80 experiments) the depressor response upon intravenous injection into the saphenous vein of solutions of these compounds was compared with acetyl choline chloride or bromide or choline chloride respectively. Solutions of equimolar concentration were employed to correct for the higher molecular weight of the perchlorate compounds. The carotid blood pressure was recorded with a mercury manometer employing ether anesthesia. Freshly prepared solutions of all compounds were injected. The results are summarized in table 1; a typical tracing is illustrated in figure 1. The choline esterase susceptibility was measured by incubation of solutions for 1 hr. at 37°C. with dog's blood serum. The depressor effect of all of these compounds was obliterated by atropinzation of the animal. Choline perchlorate possessed depressor potency equal to choline chloride.

Effect of choline esterase. Incubation for 1 hour with dog's blood serum completely inactivated solutions of acetyl choline perchlorate (fig. 2). The depressor potency of the nitrate ester of choline perchlorate, furoyl, thenoyl and benzoyl choline percolates, is not diminished by esterase activity. These observations are of theoretical interest and illustrate the importance of specific groupings in this class of compounds insofar as esterase activity is concerned.

Miosis in the rabbit. Ten animals were employed for each test. The compound was dissolved in distilled water and instilled into the conjunctival sac of one eye with massage according to the method employed by Molitor (3). The contra-lateral eye served as a control. The results are summarized in table 2.

Action on smooth muscle. The choline ester perchlorates were compared with

TABLE 1

Depressor potency of perchlorate salts of various choline esters

	PHYSICAL STABILITY IN AIR	RELATIVE DE-PRESSOR POTENCY TO A C EQUI MOLAR DOSES*	CHOLINE ESTERASE SUSCEPTIBILITY	DEPRESSOR AC TION ABOLISHED BY ATROPINE
Acetyl choline chloride	Hygroscopic	1	++	+
Acetyl choline perchlorate	Stable	1	++	+
Chloracetyl choline per-chlorate	Stable	1/500	++	+
Nitrate choline perchlorate	Stable	1/2	−	+
Acetyl-β-methyl-choline perchlorate	Stable	1	+	+
Furoyl choline perchlorate	Stable	1/5000	−	+
Thenoyl choline perchlorate	Stable	1/5000	−	+
Benzoyl choline perchlorate	Stable	1/5000	−	+
Nicotinyl choline perchlorate	Stable	1/5000	not determined	+

* The figures represent average value obtained in different animals standardized with acetyl choline chloride.
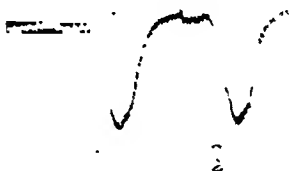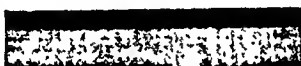

FIG 1 BLOOD PRESSURE

Dog #3, 7 kg. female. Ether anesthesia. No 1: 0 5 cc. I V. Acetyl choline chloride 1 in 10⁵. No 2: 0.5 cc. I V. Acetyl choline perchlorate equimolar solution.

the corresponding chlorides on isolated rat uterine muscle strips. Acetyl choline perchlorate was found to be equivalent to acetyl choline chloride in equimolar
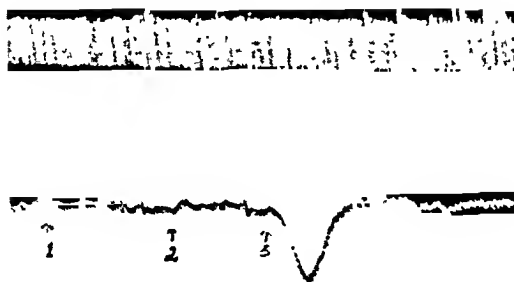
FIG. 2. EFFECT OF CHOLINE ESTERASE
Dog #3. No. 1: 0.5 cc. I. V. Acetyl choline chloride 1 in 10⁵ after 1 hr. at 37°C. with blood serum. No. 2: 0 5 cc. I. V. Acetyl choline perchlorate equimolar solution after 1 1 hr. at 37°C. with blood serum. No. 3: 0.5 cc. I. V. Nitrate ester of choline perchlorate equimolar solution after 1 hr. at 37°C. with blood serum.

TABLE 2

Miotic action of choline salts in rabbits

	PER CENT SOLUTION	AVERAGE PER CENT CONSTRUCTION OF PUPIL
Acetyl choline chloride	1	no miosis
Acetyl choline chloride	5	10
Acetyl choline perchlorate	1	no miosis
Acetyl choline perchlorate	5	10
Chloracetyl choline perchlorate	1*	no miosis
Acetyl-β-methylcholine chloride	1	60
Acetyl-β-methylcholine perchlorate	1	60
Nitrate ester choline perchlorate	1	50
Furoyl choline perchlorate	1*	no miosis
Benzoyl choline perchlorate—	0.5*	no miosis

* Limit of solubility.

concentrations (fig. 3). The furoyl, thenoyl and benzoyl perchlorates possessed approximately one-tenth the activity of acetyl choline chloride. Choline perchlorate appeared to be equivalent in potency to choline chloride and weaker

than the nitrate ester of choline perchlorate. In support of Morrison's (4) observation we found chloracetyl choline perchlorate to be about 100 times weaker than acetyl choline chloride. In these studies 70 experiments were conducted on 10 muscle preparations.
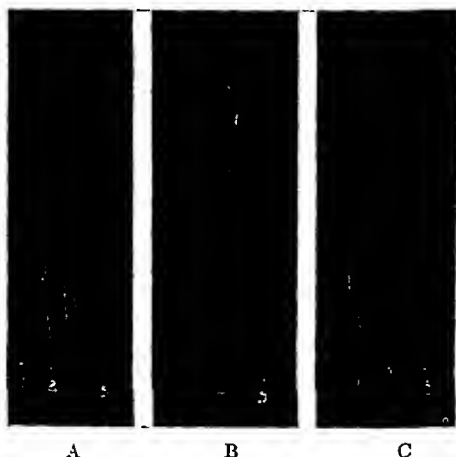

FIG. 3. EFFECT OF CHOLINE ESTERS ON RAT UTERINE STRIP

A. 1: Acetyl choline chloride 1 in 10⁵. 2: Acetyl choline perchlorate equimolar concentration. 3: Nitrate ester of choline perchlorate equimolar concentration. B 1: Acetyl choline chloride 1 in 10⁵. 2: Furoyl choline perchlorate 1 in 10⁴. 3: Thenoyl choline perchlorate 1 in 10⁴. C. 1: Choline perchlorate 1 in 10⁴. 2: Choline chloride 1 in 10⁴. 3: Nitrate ester of choline perchlorate 1 in 10⁴.

TABLE 3

Stability of choline esters in 1:1000 dilution in distilled water at 37 C.

	ORIGINAL pH	pH AFTER 14 MO	APPROXIMATE PER CENT DEPRESSOR POTENCY RETAINED AFTER		
			2 mo	4 mo	14 mo
Acetyl choline chloride-	S 2	8 0	100	100	50
Acetyl choline perchlorate—	7.8	7 S	100	80	inactive
Nitrate choline perchlorate—	8.2	9 0	100	100	50
Acetyl-β-methyl choline perchlorate-	8 0	8 2			50
Furoyl choline perchlorate	8.2	8.2	100		
Thenoyl choline perchlorate	S 4	6 2	100		

Stability. The greater physical stability of the perchlorate salts of choline and the choline esters prompted an investigation of the stability of solutions of these compounds. We were surprised to find that 1 to 1000 solutions of acetyl choline chloride were remarkably stable and even after storing in flint glass bottles at body temperature the solution retained nearly its full potency for many months and even after a year still possessed about 50% of its original

depressor activity (table 3). Higher dilutions were decomposed more rapdily (2–4 months). Acetyl choline perchlorate solutions are less stable. The decomposition of this salt is not accompanied by a change in the hydrogen-ion concentration as the pH of the solution remained the same throughout the storage period. Of interest also is the observation that 1 to 500,000 solutions of acetyl choline chloride may be boiled for 5 minutes without loss of potency.

DISCUSSION. The physical stability of the perchlorate salts of choline and its esters aids in the isolation and identification of these compounds and provides a stable salt for pharmacologic purposes. The muscarinic action of the new esters reported in this investigation compares closely with choline in potency. Chloracetyl choline perchlorate like the corresponding chloride is a relatively weak depressor and possesses feeble smooth muscle stimulating properties when tested on the isolated rat uterine strip.

SUMMARY

1. On an equimolar basis the perchlorate salts of the esters of choline possess qualitatively and quantitatively the same pharmacologic activity as the chlorides or bromides.

2. The perchlorate salts are more stable in their crystalline form than are the chlorides or bromides.

3. The pharmacology of two new esters of choline has been reported, namely, furoyl and thenoyl choline perchlorates.

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THE DISTRIBUTION OF QUINACRINE IN DOGS AND IN RABBITS

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It has been shown in the duck that the concentration of quinacrine in the plasma is extremely variable and that this variability is due to changes in the tissue/plasma distribution ratio (1). It has also been shown in the duck infected with *Plasmodium lophurae* that because of this variability the concentration of quinacrine in the plasma correlates poorly with the suppressive effect of the drug whereas there is good correlation between the suppressive effect and the dosage administered (1). Such conclusions could not be carried from the bird to man, and since it was impossible to obtain data from man, these studies of the distribution of quinacrine in dogs and rabbits were undertaken to discover whether or not the distribution of the drug in mammals was similar to that in the duck.

METHODS. Rabbits weighing from 1.4 to 2.3 kgm. were given, intramuscularly, 7 daily doses of 1 ml. per kgm. of an aqueous solution of quinacrine dihydrochloride containing 10 mgm. of quinacrine base per ml. The blood was withdrawn by heart puncture made through a cleaned area of skin and duplicate samples taken for analysis. The animals were then killed, duplicate muscle samples were taken from the right and left pectoral groups and duplicate liver samples from separate lobes.

Female dogs, weighing from 6.7 to 17.5 kgm., were given daily, by stomach tube, 10 ml. per kgm. of an aqueous solution of quinacrine dihydrochloride containing 20 mgm. of quinacrine base per 10 ml. The tubing was washed with a small quantity of water. Vomiting rarely occurred with this dose. Daily doses were omitted on the days when samples were taken. At intervals during the experiment duplicate blood samples were taken from the veins of the right and left forelegs, the dogs were anesthetized with sodium pentothal and duplicate muscle biopsies taken from the rectus abdominus or temporal muscles. At the end of the experiment a femoral vein was exposed using local anesthesia and duplicate blood samples taken. The animal was then bled to death and duplicate samples taken from the right and left pectoral muscles and from different lobes of the liver.

All samples were handled and prepared, and determinations were made as in previous work (1). Duplicate determinations on the same sample were within 5 per cent. Determinations on duplicate samples were within 30 per cent. Analyses of plasma and tissues of normal rabbits and dogs were blank for quinacrine.

It must be pointed out that in the duck and some mammals the method used determines some acridine degradation products of quinacrine (1). No similar evidence is available for the rabbit or man.

RESULTS. Data on the concentration of quinacrine in the plasma and tissues of rabbits sacrificed 24 or 48 hours after 7 daily intramuscular doses of 10 mgm. of quinacrine base per kgm. are given in table 1. Study of these data reveals that there is great individual variation in both plasma and tissue concentrations and that there is no significant correlation between them.

Table 2 gives data on the plasma and tissue concentrations of quinacrine in dogs given, by stomach tube, 20 mgm. of quinacrine base per kgm. per day

throughout the experiment. These data reveal that the concentration of quinacrine in plasma varies considerably from one dog to another and that it may rise or fall in the same dog on a constant daily dosage. The concentration of quinacrine in muscle shows considerable variation; however, the concentration of quinacrine in muscle and its ratio to the concentration in plasma tend to increase with repeated doses. There is likewise considerable variation from dog to dog in the concentration of quinacrine in the liver. In general higher liver concentrations accompany higher muscle concentrations.

TABLE 1

*Quinacrine concentration in the plasma and tissues of rabbits**

RABBIT NUMBER	PLASMA	MUSCLE	LIVER	MUSCLE PLASMA	LIVER PLASMA
Sacrificed 24 hours after last dose					
	micrograms per liter	micrograms per kgm.	micrograms per kgm.		
3678	11†	2,410	92,600	219	8,410
3689	13†	6,560	205,000	505	15,800
3677	40	25,200	278,000	631	6,960
3695	44	5,200	208,000	118	4,700
3687	56	8,910	140,000	159	2,500
3688	68	5,710	118,000	84	1,730
3682	76	8,490	218,000	112	2,870
Sacrificed 48 hours after last dose					
3683	10	4,410	213,000	441	21,300
3696	22	11,400	244,000	518	11,100
3686	24	5,310	107,000	222	4,450
3693	34	3,650	60,000	107	1,770
3694	37	9,130	150,000	247	4,050
3685	43	3,430†	124,000	80	2,880
3684	83	15,500	262,000	187	3,160

* All rabbits received, intramuscularly, 10 mgm. quinacrine base per kgm. body weight per day for 7 days.

† Analyses of duplicate samples differed by more than 30 but less than 50 per cent.

DISCUSSION. The data given here show that the tissue/plasma distribution ratios of quinacrine in the dog and rabbit are of the same order of magnitude as in the duck (1). It seems probable that this is also true of the rat (2). There is no significant correlation between the concentration of quinacrine in the plasma and that in the tissues in dogs, rabbits or ducks; however, in dogs (3) and in ducks (1) the concentration of quinacrine in the tissues is apparently directly related to the dosage. In ducks, dogs or rabbits large variations in the concentration of quinacrine in the plasma can occur as a result of changes in distribution producing insignificant alterations in the concentration of the drug in the tissues. This situation is reflected by the large individual variations found, in these

experiments, in the concentration of quinacrine in the plasma in members of the same species given equal doses of the drug   Similar individual variations of the concentration of quinacrine in human plasma have been observed (4, 5, 6). In view of the essential similarity of the distribution of quinacrine in the avian

TABLE 2

*Quinacrine concentrations in the plasma and tissues of dogs**

DOG NO	NO DAILY DOSES	PLASMA	MUSCLE	LIVER	$\dfrac{\text{MUSCLE}}{\text{PLASMA}}$	$\dfrac{\text{LIVER}}{\text{PLASMA}}$
		micrograms per liter	*micrograms per kgm*	*micrograms per kgm*		
1	7	70	6,380		911	
	20	81	13,900		171	
	35	60	11,300		188	
	44	62				
	65	28				
	80	28	30,500	241,000	1,090	8,600
2	14	72	20,500		285	
	40	54	12,600		233	
	55	57	38,500	303,000	675	5,300
3	7	70	7,560		108	
	20	95	17,500		184	
	42	406	126,000	475,000	310	1,170
4	14	209	15,900		76	
	40	237	24,000		102	
	55	204	124,000	2,240,000	608	11,000
5	7	69	9,830		142	
	26	69	10,600		154	
	37	80				
	51	50	27,400	166 000	548	3,320
6	14	87	7,500		86	
	34	136	11 200		82	
	38	69				
	66	46				
	72	59	44,000	1,010,000	745	17,100

* All dogs received, by stomach tube, 20 mgm quinacrine base per kgm body weight per day throughout the experimental period except for days on which samples were taken They were sacrificed 24 hours after the last dose

† Analyses on duplicate samples differed by more than 30 but less than 50 per cent

and mammalian species studied and because of the similar individual variations in the concentration of quinacrine in the plasma of animals and man, it seems probable that the distribution of quinacrine in man follows the same pattern as in animals   If this is so, one would expect to predict the suppressive effect more accurately on the basis of dosage than on the basis of the concentration of

quinacrine in the plasma.   The correlation of suppressive effect with dosage has been shown by Fairley (4).

SUMMARY

The concentration of quinacrine in the plasma of rabbits and dogs shows marked variation.   There is no significant correlation between the concentration of quinacrine in the plasma and that in the tissues of rabbits and dogs.   The concentration of quinacrine in muscle and its ratio to the concentration in the plasma tend to increase in dogs on a constant daily dosage.

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STUDIES IN VITRO AND IN VIVO ON THE INFLUENCE OF THE LIVER ON ISONIPECAINE (DEMEROL) ACTIVITY

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Very little is known about the absorption, fate and excretion of isonipecaine (demerol, meperidine) although the compound is widely used as an analgetic, especially in the field of obstetrics. The excretion of isonipecaine has been studied by Lehman (1) and Oberst (2). Bernheim (3) has reported that isonipecaine is hydrolyzed in vitro by the liver of various animals but not by brain, blood, kidney, spleen or heart.

Negative in vitro results do not necessarily imply that such is always the case in vivo. The findings may merely mean that certain conditions of the experiment, such as enzyme or substrate concentration, temperature, pH, etc., were not optimal for tissue activity. Consequently, we decided to study the effects of various tissues on isonipecaine in vitro under conditions different from those used by Bernheim. It was possible for us, by modifying Brodie and Udenfriend's methyl orange method for cinchonidine (4), to follow the fate of very low concentrations of isonipecaine. To substantiate our in vitro findings, we followed up with in vivo experiments.

EXPERIMENTAL. Known amounts of isonipecaine were added to buffered aqueous suspensions of minced rat, dog or human tissues and after incubating for fifteen minutes to three hours, the isonipecaine was determined in duplicate by a modification of Brodie and Udenfriend's method (4). A smaller volume of ethylene dichloride was employed so that we could facilitate readings in a Coleman Junior Spectrophotometer model 6A instead of the Evelyn. Blank values were first obtained on tissue suspensions containing no isonipecaine. By using a M/1 phosphate buffer of pH 7 to wash the alkaline ethylene dichloride extract of tissue, it was possible to decrease blank readings to an optical density of 0.015 or less. Inasmuch as this buffer wash does not decrease isonipecaine readings, greater specificity in the methd is obtained in addition to the increased accuracy at low values.

Reagents. The reagents were prepared as outlined by Brodie (4). Standard Solution: A master standard solution of isonipecaine was prepared by dissolving 114.8 mg. of the hydrochloride in 1 liter of distilled water. Working standards of 20, 10, 5, and 2 mg./liter were made from the master standard. When concentration was plotted against optical density, a straight line was obtained. No change in readings was noted in the standards left at room temperature for several months.

Procedure. A weighed sample of tissue (1 gm , whenever possible) was added to 10, 15, or 20 cc. of 0.1M phosphate of pH 7.5 and minced in a Waring Blendor. After adding 1 cc. of 20 mg. per liter isonipecaine to 1 cc. of tissue suspension in a glass stoppered 50 cc. Erlenmeyer and incubating for a fixed interval at 38°, 1 cc. of N/1 NaOH and 10 cc. of ethylene dichloride were added, and thorough mixing effected by placing the flask on a mechanical shaker for 5 minutes The flask contents were transferred to a pyrex ignition tube (16 × 120 mm), centrifuged for 5 minutes, and the supernatant aqueous layer removed by aspiration. The ethylene dichloride was then placed in the flask containing 5 cc. of M/1 phosphate

huffer. The contents were thoroughly mixed by shaking for five minutes, transferred to a pyrex ignition tube and centrifuged five minutes. The supernatant layer was completely removed by aspiration and the ethylene dichloride decanted into shaking flasks containing 0.5 cc. of methyl orange. After shaking for three minutes, the excess methyl orange was carefully removed by centrifuging and aspiration. After recentrifuging the ethylene dichloride, 5 cc. were pipetted into a colorimeter tube containing 1 cc. of alcoholic H_2SO_4. The color developed was read in the Coleman Junior at 540 mu. with distilled water being used to set the instrument at zero optical density. After correcting for the tissue blank, the concentration of isonipecaine present was obtained by reference to the standard curve. An optical density of 0.39 is obtained when twenty micrograms of isonipecaine is run through the procedure described above.

RESULTS. In experiments using the organs of approximately one hundred rats under various conditions, the liver was found to be by far the most active
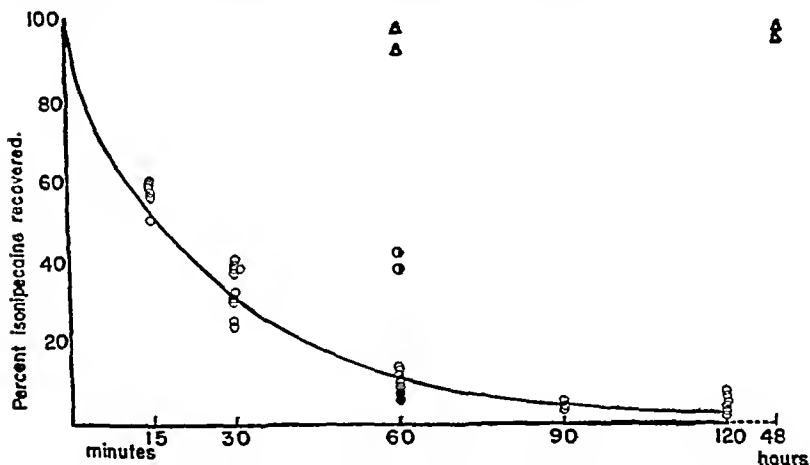

FIG. 1. RATE OF INACTIVATION OF ISONIPECAINE BY O RAT LIVER, Δ RAT LIVER IN M/50 NaF, ● DOG LIVER, AND HUMAN LIVER ◑

organ for destroying isonipecaine. On incubating twenty micrograms of isonipecaine with fifty to two hundred milligrams of minced liver practically no isonipecaine could be detected after ninety minutes. Fresh liver specimens had to be used as the liver rapidly lost most of its activity on standing at room temperature for three hours. The activity can be preserved to a great extent by storage at −20 degrees. No loss in potency was noted in liver perfused free of blood. The data obtained from the liver of five rats are shown in figure 1. The reaction seems to be unimolecular and can be inhibited with M/50 sodium fluoride. The liver of two dogs showed activity comparable to rat liver in its ability to destroy isonipecaine, and its activity was also lost on standing. The minced liver of one man and one woman obtained at autopsy three to five hours after death had occurred showed only slight ability to decrease isonipecaine recovery, but liver slices of one human obtained at biopsy were quite active. The three hour old

liver preparations of rats, dogs and humans, which no longer destroyed isonipecaine, were still quite active for hydrolyzing acetylsalicylic acid (5). It appears, therefore, that the two compounds do not have a common enzyme for their inactivation.

In earlier experiments with rat blood, either oxalated, heparinized or defibrinated, very slight or no loss in isonipecaine was obtained after an incubation period of sixty minutes or less. However, in later experiments when the incubation period was increased to ninety or more minutes, rat blood showed some ability to decrease isonipecaine values.

This activity was inhibited by M/50 NaF. The blood of two dogs and three humans did not exhibit the activity found in rat blood. Also, no decrease in
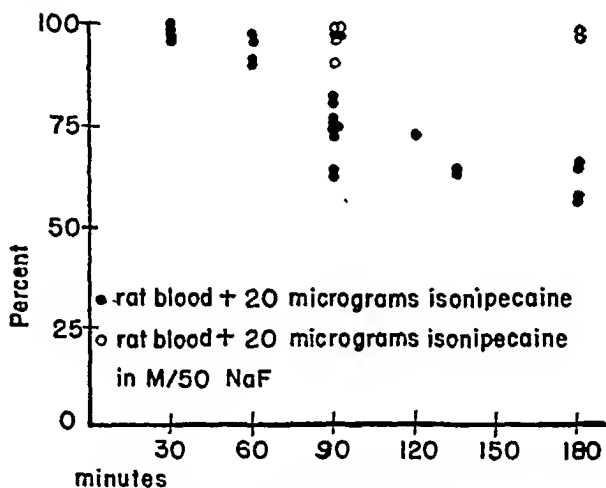
FIG. 2. PER CENT ISONIPECAINE RECOVERED FROM 1cc. RAT BLOOD AFTER INCUBATION AT 38°

isonipecaine was noted on incubating the compound twenty-four hours with phosphate buffered to a pH of 7.5 (fig. 2).

In earlier experiments, rat lung was found to decrease the amount of isonipecaine recovered but this activity was about the same order of that of rat blood. Subsequent experiments on the lungs of four rats perfused free of blood indicated no appreciable activity present. Other rat tissues, brain, heart, spleen, muscle, and kidney, showed little if any ability to decrease isonipecaine values after one hour of incubation.

Attempts were then made in vivo to establish evidence confirming the in vitro findings that the liver inactivates isonipecaine. We have previously found that isonipecaine potentiates barbiturate depression (6) It has also been shown that thiopental (pentothal) differs from other short acting barbiturates like evipal or seconal in that its hypnotic effect is not materially increased after ex-

perimental liver damage (7) or partial hepatectomy (8, 9). If it can be demonstrated that partial hepatectomy causes a considerable increase in the duration of sleep in animals given thiopental plus isonipecaine, then the liver must be essential for the inactivation of isonipecaine. Before attempting this experiment we decided to confirm first the previous findings on thiopental and evipal (8, 9).

METHOD. In three series of rats, evipal (70 mg./kg.), thiopental (30 mg./kg.), or thiopental (30 mg./kg.) plus isonipecaine (20 mg./kg.) were injected intraperitoneally and the duration of hypnosis measured. An animal was considered to be awake when it could right itself after being pinched on the tail. The rats were then anesthetized with ether and approximately two-thirds of the liver was removed in the manner described by Higgins (10). After forty to forty-eight hours, the rats were again given the same intraperitoneal dosage of evipal, thiopental, or thiopental plus isonipecaine and the duration of sleep determined. Two to five weeks were allowed for liver regeneration and the duration of hypnosis was again determined with the same drugs. The average duration of hypnosis was also determined in six rats which received thiopental (30 mg./kg.) plus isonipecaine (60 mg./kg.).

In another experiment, a comparison of the blood levels of isonipecaine was made on partially hepatectomized and normal rats. Isonipecaine, 40 mg./kg., was injected intraperitoneally into eight normal rats and into eight partially hepatectomized rats forty-eight hours after the completion of the operation. One hour after giving the drug, blood was obtained by cardiac puncture, and one cubic centimeter samples were assayed for isonipecaine by the method described earlier.

RESULTS. Our findings on evipal and thiopental are in agreement with previous reports (7, 8, 9). As can be ascertained from figure 3, the average duration of evipal (70 mg./kg.) induced hypnosis increased from eleven minutes to fifty-three minutes forty-eight hours after partial hepatectomy. After a twelve day interval to allow the liver to regenerate, the average duration of evipal hypnosis was thirteen minutes.

With thiopental, partial hepatectomy increased the duration of hypnosis only from nineteen minutes to twenty-eight minutes, whereas, with thiopental plus isonipecaine (20 mg./kg.), four out of eleven rats died, and the duration of sleep in the survivors increased from thirty-seven minutes to ninety-one minutes. Two to five weeks later, when the same dosage of each compound was again administered, the duration of hypnosis approximated the normal preoperative values.

With the same dose of thiopental (20 mg./kg.) plus three times the dose of isonipecaine (60 mg./kg.) in normal rats, two out of six rats died and the average duration of sleep for the survivors averaged more than eighty minutes.

The blood levels of isonipecaine determined on rats are presented in figure 4. Many of the values were obtained at the lowest range of sensitivity of the Coleman spectrophotometer; consequently, the exact value of an individual low reading may be questioned. Nevertheless, the method was sufficiently precise
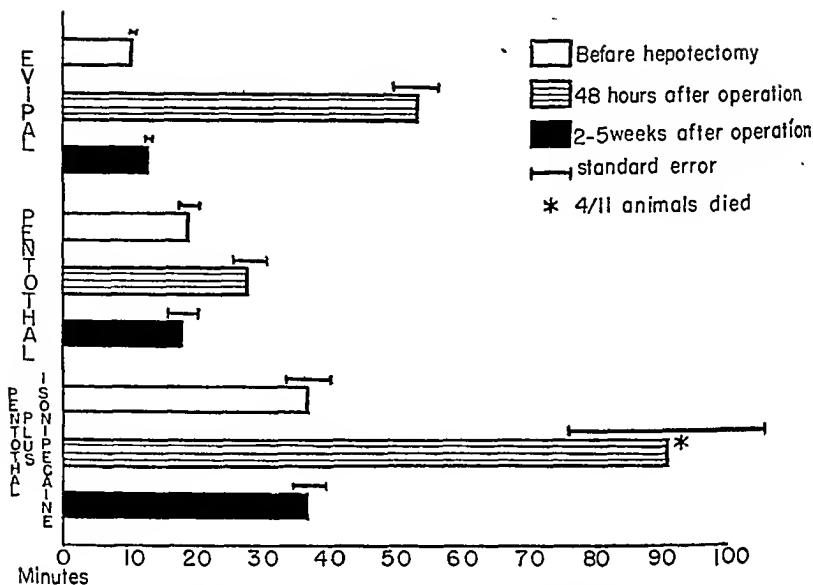
FIG. 3. EFFECT OF PARTIAL HEPATECTOMY ON THE DURATION OF HYPNOSIS IN WHITE RATS GIVEN EVIPAL, PENTOTHAL OR PENTOTHAL PLUS ISONIPECAINE
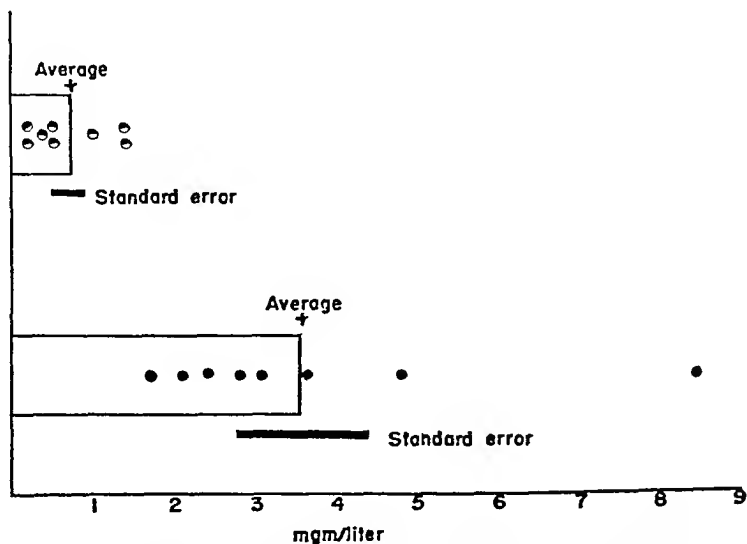

FIG. 4. BLOOD LEVEL OF ISONIPECAINE IN ◓ NORMAL AND ● PARTIALLY HEPATECTOMIZED RATS ONE HOUR AFTER 40 MGM./KGM. INTRAPERITONEALL

(optical density 20 micrograms isonipecaine by method read 0.39) to indicate that the blood levels of isonipecaine in the eight partially hepatectomized rats were consistently higher than those in the eight normals. The blood level for normal rats one hour after isonipecaine, 40 mg./kg., intraperitoneally, averaged $0.7 \pm .2$ (standard error) mg./liter, whereas it averaged $3.55 \pm .8$ mg./liter in the operated animals. The probability that the difference in the two values occurred by chance is less than 1 in 100 (calculated P less than 0.01).

DISCUSSION. Since partial hepatectomy does not increase the duration of thiopental hypnosis to any great extent, it seems reasonable to conclude that the increased effect obtained with isonipecaine plus thiopental after partial hepatectomy is due mainly to the inability of the impaired liver to inactivate isonipecaine. This is further substantiated by the fact that the duration of thiopental-isonipecaine hypnosis can be prolonged by an increase in isonipecaine dosage, and by the fact that with equivalent doses of isonipecaine, the blood levels of the compound in the partially hepatectomized rats were found to be consistently higher than the levels in the normal animals.

Although the point of whether or not the liver destroys thiopental is in dispute, this does not interfere with the validity of our results. Recently, investigators (11, 12) have reported that the liver is quite active in destroying thiopental but the fact remains, that under the conditions of our experiments, partial hepatectomy does not materially increase the duration of action of thiopental. (Goldbaum (11) has found that other tissues in addition to the liver inactivate thiopental in vitro. He suggests that even when a large portion of the liver is removed, no great increase in thiopental effects is obtained because other tissues are still able to destroy the compound. Richards (13) has reported that the blood inactivates thiopental.)

It is of importance to apply the above findings to the clinical usage of isonipecaine. Should it be necessary to use the compound in patients with severe hepatic impairment, caution must be exercised. It is highly probable that smaller doses of isonipecaine than that usually employed can be administered to obtain the desired therapeutic effect in such individuals.

SUMMARY

1. Evidence that the liver inactivates isonipecaine is indicated by the fact that effects and blood level of isonipecaine are increased in rats after partial hepatectomy.

2. In vitro findings on rat, dog and human liver support the in vivo results.

3. Under similar conditions in vitro, other tissues exhibited slight or no activity.

4. It is suggested that in patients with severe hepatic impairment, the desired therapeutic effect of isonipecaine can be obtained with smaller dosages.

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THE QUANTITATIVE ESTIMATION OF THEOPHYLLINE IN BLOOD[1]

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Although the analytical methods for the methylated xanthines used in medicine are legion, our search of the literature revealed no satisfactory method which was applicable to body fluids. Recently our interest in the determination of blood levels for theophylline was aroused by the preparation of a new theophylline derivative in this laboratory, namely, theophylline and sodium aminoacetate (1). In addition, the development of a rapid analytical method for theophylline in the blood would enable one to compare rates of absorption and dissipation of the various theophylline derivatives, and furthermore, adequate therapy in terms of blood levels for asthma and post-coronary conditions could be established.

PRELIMINARY TRIALS. Many of the characteristic oxidative reactions for the xanthine bases were found unsuited owing to the lack of quantitative character of the reaction and low sensitivity. A method developed in the laboratory of Parke, Davis and Company (2) which depended upon the use of Gibbs' reagent (2,6-dichloroquinone-chloroimide) for phenol was not satisfactory in our hands owing to the multiplicity of structures which give positive reactions with this reagent. After extraction from blood filtrates with chloroform, evaporation and redissolving the theophylline in water, we were able to determine this xanthine with a fair degree of accuracy spectrophotometrically. The method, however, is arduous and lengthy, and many extraneous substances frequently interfered with its validity. We therefore sought further for a suitable chromogenic reaction that might simplify the procedure.

CHEMICAL CONSIDERATIONS. Theophylline will undergo azo coupling with diazotized sulfanilic acid (3); however, the reaction is not sensitive and must be carried out with the dry compounds. Sanchez (4) found that by treating the methylated xanthine derivatives with strong alkali, azo linkage was more readily effected. We repeated Sanchez' work, who used diazo-p-nitroaniline as a coupling reagent. We found that this reaction too lacked sensitivity. Dr. W. Minnis of the National Aniline Division of the Allied Chemical and Dye Corporation, sent us numerous samples of stabilized double diazonium and zinc chloride salts and we proceeded to explore these as possibilities. The "Fast Blue 2 B Salt", which is the stable zinc chloride complex of the diazotized 5-amino-2-benzoylamino-1,4-diethoxybenzene, was found to react with the alkali decomposi-

tion product of theophylline in very high dilution. Theobromine, caffeine, uric acid, creatinine, creatine, urea and glycine, subjected to the previous alkali treatment did not interfere with the test. These compounds in comparable dilutions produced no color at all or compounds exhibiting a different color, easily extinguished by appropriate filters. We decided therefore to explore this reaction further.

When boiled with concentrated fixed alkali solutions, Maly and Andreasch (5) give the following degradation of caffeine to form caffeidine:


Caffeine   Caffeidine
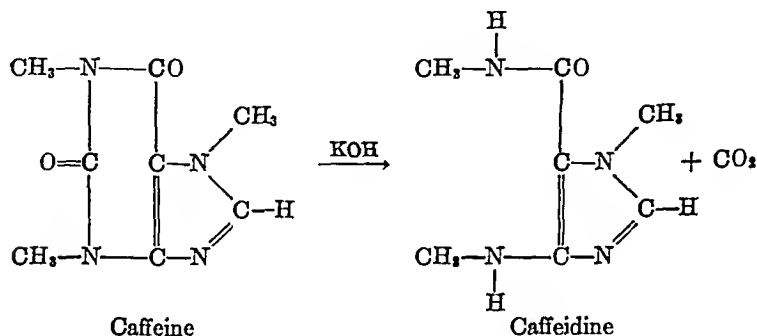
Prolonged heating will further decompose the caffeidine to methylamine and other decomposition products

Presumably, theophylline which is 1,3-dimethylxanthine, undergoes a similar reaction in the presence of alkali to form the analogous theophyllidine.

The coupling reaction for theophylline produces a comparatively stable red color readily detectable in dilutions of 0 1 mg. per cent when 5 cc. of theophylline solution is employed.

In our opinion the reaction proceeds according to the following equation:

"Theophyllidine"   Fast Blue 2 B Salt
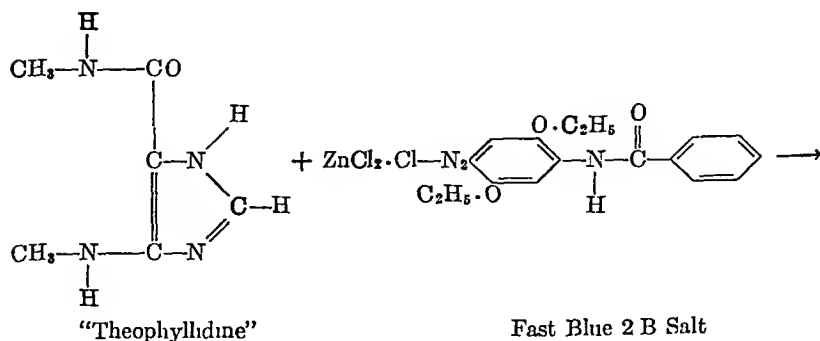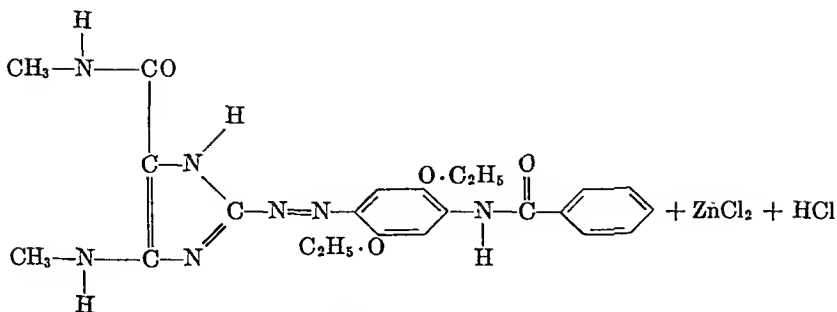
Red Azo Derivative

To determine the time of maximal color formation, readings were taken at one minute intervals in the electrophotometer. The optimum reading should be made between five and six minutes after the addition of the coupling reagent.

COLOR VERSUS CONCENTRATION. Several concentrations of theophylline in water were subjected to the determination and a standard curve for aqueous solutions constructed as shown in figure 1.

A similar curve was constructed using oxalated pooled human blood. These data are shown in figure 2.

Each point on the curve is the result of 10 or more determinations on known theophylline concentrations in blood.

The procedure for blood is carried out as follows:

1. Add 5 cc. of oxalated blood to a mixture of 10 cc. of water, and 5 cc. of 2/3 normal sulfuric acid contained in a 50 cc. centrifuge tube. Mix thoroughly.
2. When the blood is laked add 5 cc. of 10 per cent sodium tungstate solution and mix thoroughly.
3. Centrifuge for 10 minutes at 2,500 R.P.M.
4. Decant the supernatant liquid and filter through a 12.5 cm. No. 2 Whatman filter.
5. Evaporate 10 cc. of the clear filtrate to dryness in a glass evaporating dish on a steam bath aided by a current of air.
6. Extract the residue with 3 successive 5 cc. portions of chloroform.
7. Filter the combined chloroform extract through a pledget of cotton into a tube of thin pyrex glass having a diameter of 25 mm. and a length of 100 mm.
8. Evaporate the chloroform on a steam bath.
9. Add exactly 2 cc. of 50 per cent W/V potassium hydroxide solution; add a glass bead, heat to boiling and boil for exactly 20 seconds over an open flame.
10. Immerse the tube in a beaker of cold water (15 to 20° C.) for 3 minutes.
11. Add exactly the quantity of 50 per cent V/V glacial acetic acid required to neutralize the potassium hydroxide used, as determined previously by titration, using methyl red as an indicator.
12. Cool the solution to room temperature by immersing in cold water; make up to 5 cc. with water and add 5 cc. of the Fast Blue 2 B Salt reagent[2] and mix.

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[2] The reagent is prepared by dissolving 100 mg. of Fast Blue 2 B Salt in 100 cc. of isopropanol (Merck's Reagent Grade). Filter through dry paper and preserve at 0°C. The reagent should be prepared freshly each 2 hours as even at 0°C. it spontaneously undergoes decomposition.
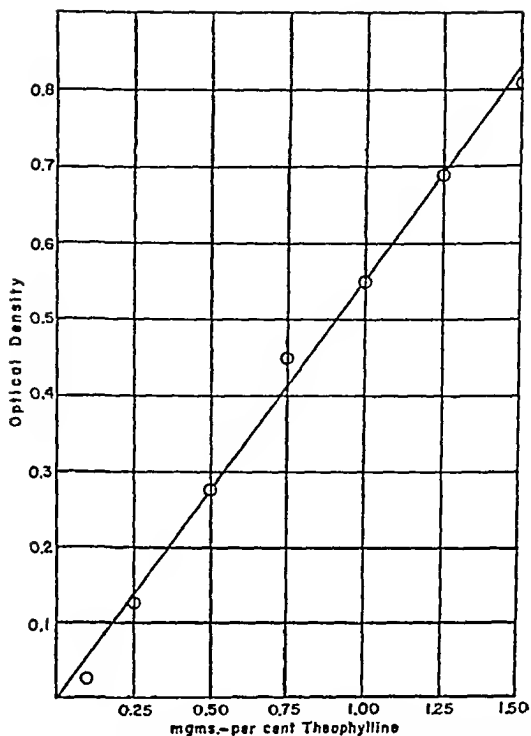
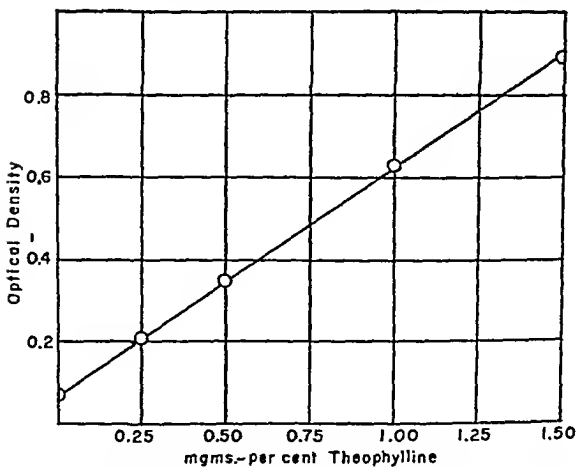FIG. 1. CONCENTRATIONS OF THEOPHYLLINE AND OPTICAL DENSITY

FIG. 2. BLOOD LEVELS OF THEOPHYLLINE AND OPTICAL DENSITY

13. At 5 to 7 minutes read the color intensity in a Fisher electrophotometer, using a 525 mu filter and 5 cc. cell.

In this laboratory the method required 45 minutes for one determination and 15 minutes additional for each duplicate sample.

RESULTS ON HUMAN BLOOD OF KNOWN THEOPHYLLINE LEVELS. Using pooled human blood, definite amounts of anhydrous theophylline were added and subjected to the foregoing determination. The data are shown in table 1.

APPLICATION OF METHOD TO THEOPHYLLINE THERAPY. Bloods from several individuals receiving theophylline therapy in the Allergy Clinic of one of us (H. M. B.) of this institution were drawn and subjected to analysis.[3] Bloods from eight patients receiving 1 to 2 Gm. of theophylline per day (in the form of theophylline sodium aminoacetate) were examined. They were found to contain 0.23 to 1.8 mg. per cent. Further studies in therapy are in progress.

TABLE 1

Theophylline in human blood

SAMPLE NUMBER	KNOWN THEOPHYLLINE	FOUND	DIFFERENCE
	mg. per cent	*mg. per cent*	*mg.*
1	0.20	0.24	+0.04
2	0.20	0.18	−0.02
3	0.40	0.40	0.00
4	0.40	0.39	−0.01
5	0.60	0.57	−0.03
6	0.80	0.83	+0.03
7	0.80	0.82	+0.02

SUMMARY

A colorimetric method for the quantitative estimation of theophylline in blood has been developed. It appears to be applicable to theophylline-blood levels of patients receiving theophylline therapy.

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[3] We are indebted to Dr. Sally Cook for assisting in these clinical studies.

LOCAL ANESTHETIC ACTION OF A SERIES OF
ARYL-URETHANES

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A series of thirteen compounds possessing local anesthetic action has been developed[1]; these are aryl-urethanes of two groups conforming to the formulae below and varying in the length of the carbon chain indicated by R.
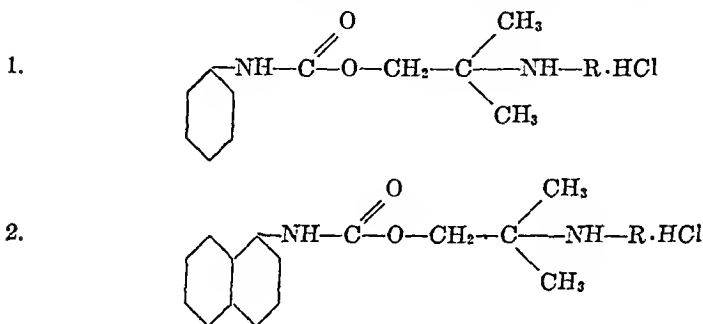
In this paper the members of the first of these groups will be referred to by the code numbers Ur-21, Ur-22, etc., and those of the second group as α-21, α-22, etc. Several related compounds (phenylurethans) with local anesthetic properties have been described (1, 2).

The local anesthetic effectiveness of these compounds was compared with that of cocaine hydrochloride as a standard by measuring their ability to suppress the corneal reflex of the rabbit eye, according to a modification of the method of Schmitz and Loevenhart (3). After preliminary trials of various dilutions the new compounds were used at a concentration of 0.1 per cent and the cocaine at 1 0 per cent. The right eye of each animal received 5 drops of the test solution which was washed out thoroughly after 3 minutes, and the left eye was treated with cocaine in the same manner. The corneal reflex was tested at regular intervals thereafter; observations were also made on pupillary size and the occurrence of edema, inflammation and corneal pitting Five rabbits were used for each substance investigated Two ratios were established in each case, (a) that between the duration of effect of the test drug and that of cocaine at the time when recovery of the reflex began to occur and (b) that between the duration of effect of the two drugs when the reflex was fully re-established.

The results of this experiment bearing on anesthetic activity are tabulated in Table I. All of the aryl-urethane compounds are shown to possess local anesthetic action. Since the cocaine solution was of a concentration ten times

that of the test materials, it would appear from the ratios shown in the table that all of these compounds are appreciably more active than cocaine in terms of duration of effect. This becomes most striking in the case of the compounds with the longer substituted alkyl groups.

TABLE I

Comparative duration of anesthesia of the rabbit eye produced by cocaine (1%) and some aryl-urethanes (0.1%)

Code number	Carbon chain	BEGINNING RECOVERY				COMPLETE RECOVERY			
		Duration	Difference in duration	P-value* for difference	Ratio of experimental:control	Duration	Difference in duration	P-value for difference	Ratio of experimental:control
		min.				*min.*			
Ur-21	$-CH_2CH_3$	20	-12	.03	.63	85	3	.69	1.04
Cocaine		32				82			
Ur-22	$-CH_2CH_2CH_3$	37	-2	.70	.95	93	3	.31	1.03
Cocaine		39				90			
Ur-23	$-CH_2CH_2CH_2CH_3$	34	0	1.00	1.00	90	13	.19	1.17
Cocaine		34				77			
Ur-24	$-CH_2CH_2CH_2CH_2CH_3$	45	6	.10	1.15	115	22	.002	1.24
Cocaine		39				93			
Ur-25	$-CH_2CH_2CH_2CH_2CH_2CH_3$	69	30	.04	1.77	133	28	.04	1.27
Cocaine		39				105			
Ur-26	$-CH_2CH_2CH_2CH_2CH_2CH_2CH_3$	96	64	.004	3.00	165	64	<.002	1.63
Cocaine		32				101			
Ur-29	$-CH_2CH_2CH\genfrac{}{}{0pt}{}{CH_3}{CH_3}$	65	31	.02	1.91	178	46	<.002	1.35
Cocaine		34				132			
α-21	$-CH_2CH_3$	41	1	.95	1.03	109	18	.03	1.20
Cocaine		40				91			
α-22	$-CH_2CH_2CH_3$	44	13	.41	1.42	85	16	.004	1.23
Cocaine		31				69			
α-23	$-CH_2CH_2CH_2CH_3$	89	54	<.002	2.54	184	71	.01	1.63
Cocaine		35				113			
α-24	$-CH_2CH_2CH_2CH_2CH_3$	177	136	<.002	4.32	259	146	<.002	2.29
Cocaine		41				113			
α-25	$-CH_2CH_2CH_2CH_2CH_2CH_3$	173	136	<.002	4.68	271	162	<.002	2.49
Cocaine		37				109			
α-26	$-CH_2CH_2CH_2CH_2CH_2CH_2CH_3$	190	156	<.002	5.59	308	211	<.002	3.18
Cocaine		34				97			

* A P-value of .05 or less indicates statistical significance.

Ur-29 is a five-carbon branched chain compound rather than a straight chain compound like the others.

In both groups of drugs a general correlation appears to exist between length of the carbon chain and the anesthetic effect. This relationship is shown in figures 1 and 2 in which duration of effect of the compounds is plotted against
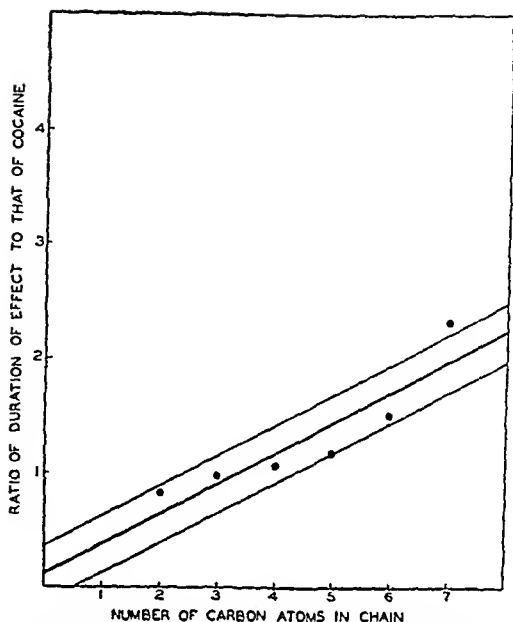
FIG. 1. LINE OF REGRESSION FOR RATIO OF DURATION OF EFFECT OF COMPOUNDS OF *Ur* SERIES TO DURATION OF EFFECT OF COCAINE
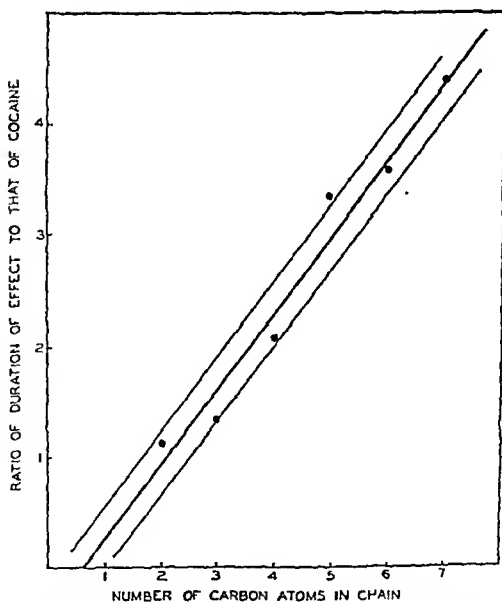

FIG. 2. LINE OF REGRESSION FOR RATIO OF DURATION OF EFFECT OF COMPOUNDS OF *α* SERIES TO DURATION OF EFFECT OF COCAINE

the number of carbon atoms in the chain. The standard error for the line of regression is indicated by the lighter lines parallel to it.

In general there was also a correlation between solubility of the compounds in water and their anesthetic effects, the more difficultly soluble substances being more effective.

The occurrence of the side reactions in the series is tabulated below:

	INFLAMMATION	EDEMA	PITTING	PUPILLARY SIZE
Cocaine..	slight	none	marked	marked increase
Ur-21....	slight	none	none	no change
Ur-22...	none	none	none	no change
Ur-23.............	none	none	slight	slight increase
Ur-24.............	slight	none	slight	no change
Ur-25.............	moderate	none	slight	slight increase
Ur-26.............	slight	none	slight	no change
Ur-29.............	very slight	none	none	no change
o-21.............	none	none	none	no change
α-22.....	none	none	none	no change
α-23............	slight	none	slight	no change
α-24.............	none	none	slight	no change
α-25.............	none	none	slight	no change
α-26.	slight	none	slight	no change

SUMMARY

The local anesthetic action of a series of aryl-urethane compounds has been tested. All compounds appeared to possess anesthetic activity greater than that of cocaine hydrochloride, this difference being most marked in the case of the compounds with the longer substituted alkyl groups. A correlation was shown to exist between the duration of effect and the length of the substituted alkyl group in each of the two series.

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STUDIES IN ANALGESIA: PIPERIDINE DERIVATIVES WITH MORPHINE-LIKE ACTIVITY

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In the search for a better analgesic agent than morphine most attempts of the past have been directed toward modifying the morphine molecule or breaking it down to find some constituent part of its structure responsible for the analgesic action. Only closely related derivatives of morphine maintained the analgesic action. Unfortunately these compounds also possessed a considerable degree of tolerance and addiction potentiality, e.g., heroine, dilaudid, metopon, desomorphine. Until the introduction of the piperidine derivative Demerol (Dolantin) in 1939 by Eisleb and Schaumann (1) no substance was known which approached morphine or its derivatives in pain-relieving action. The piperidine nucleus in the form of N-methyl piperidine may be identified in the morphine molecule but this fact in itself is not significant since this nucleus is found in a number of non-analgesic alkaloids, e.g., atropine, lobeline. The major part of the Demerol structure can be found in the morphine molecule but one can hardly affirm that this accounts for the analgesic activity of morphine since the latter is more active than Demerol and also since the most active compound reported here, Nu-718 an isomer of Demerol, structurally resembles morphine slightly less than does Demerol, although it is thirty times more active than the latter.

It is now well established (2, 3, 4) that Demerol can produce tolerance and addiction. It is apparent therefor that these undesirable effects are not peculiar to the morphine molecule. It is possible they may be irrevocably tied up with analgesia itself. In the search for better analgesics the problem is not just to find a substance more potent than any preexisting compound although this is the most direct line of attack in the laboratory, but rather one of also finding a compound devoid of or at least with greatly diminished side effects, particularly tolerance and addiction. However, this goal can not be successfully evaluated in the laboratory so the laboratory problem does necessarily become one of searching for a potent analgesic whose chemical structure differs from other analgesics in the hope that it will later be found to possess greatly reduced tolerance and addiction liability, as well as a reduction in other side effects.

This report gives the results of screening tests on the first series of new compounds synthesized (5). The study has included chiefly analgesia and toxicity with a limited amount of other pharmacological data.

METHOD OF TESTING ANALGESIA. Rats were employed as the test animal by a method basically that of Hardy, Wolff, and Goodell (6). Ercoli and Lewis (7) described a modification which we used after making the following changes: A higher wattage projection

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lamp (1500 W., 110 V.) was used but operated at a lower voltage (60 V.). A longer life with slower changes in the radiation characteristics was thus attained and a greater proportion of heat to light waves was produced. We also designed a light aluminum shutter the operation of which was automatically synchronized with the stop-watch. In brief, the apparatus set-up consisted in focussing the light from the lamp through a 4 inch diameter, 9 cm. focal length bi-convex lens at an 18 mm. diameter aperture in a lucite screen placed about 38 cm. from the lamp filament. Voltage was controlled by a Varitran transformer. The test-animal, after removal of hair by clipping, was held vertically, head up, with its back against the aperture in the lucite screen. After the animal became quiet the shutter was opened and the reaction time measured to the first appearance of skin twitching.

Rats weighing from 150 to 250 grams were employed in groups of five. Control reaction times were determined by averaging three tests run at 15 minute intervals before injection. The normal reaction time was usually about 4 seconds. Subsequent to injection, tests were made at half hour intervals until the reaction time returned to normal. The animals were used not oftener than once a week.

To describe the results quantitatively on a basis independent of the actual reaction times an analgesic index was devised which would provide a numerical value of the potency and also reflect the relation between dosage and effect. The formula,

$$\text{Analgesic index} = \sqrt{\frac{t_2}{t_1}}$$

was employed, where t_1 is the normal reaction time in seconds and t_2 the altered reaction time. This square root formula has given figures expressing potency which when plotted against the dose give approximately straight line curves.
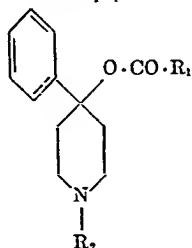
EXPERIMENTAL RESULTS. The results on toxicity and analgesia are given in Tables I to III. The compounds are tabulated in order of their laboratory code numbers. The most important group, the eleven 1-alkyl-4-phenyl-4-acyloxy piperidine derivatives have been segregated and placed at the beginning of Table I. Type structural formulas are given at the head of each table.

The compounds were administered as the hydrochloride salts but are reported in terms of free bases except in Table VI. Toxicities were determined by sub-cutaneous injection in mice and the LD 50's together with the number of animals used are tabulated. Relatively few mice were used in most cases since the experiments were mainly for screening purposes. Also in some cases the amount of material available was small.

The analgesic action of the compounds is compared at a constant effect. For a standard reference level an analgesic index of 1.40 was chosen. This represents about twice the normal reaction time. The dose giving this index is termed the AD 1.40. It was estimated by plotting the indices against the doses. The AD 1.40's together with the number of rats employed are listed in the tables. It was considered worthwhile to list the actual analgesic indices obtained with doses which were approximately one-third the mouse LD 50 (mgm./kgm. basis). If a compound does not produce significant analgesia at one-third the mouse LD 50 there is little likelihood that it could be clinically important in the relief of severe pain.

The standard reference level of 1.40 was selected also on the basis that beyond this value rats very rapidly develop complete analgesia. With complete anal-

TABLE I

1-alkyl-4,4-substituted piperidine compounds

COMPOUND NUMBER	FORMULA	MOUSE SUBCUTANEOUS LD50	N	RAT ANALGESIC DOSE AD 1.40	N	ANALGESIC INDEX AT ABOUT ½ MOUSE LD50
		mgm./kgm.		*mgm./kgm.*		
Nu-718	1-methyl-4-phenyl-4-propionoxy-piperidine	40	12	1.4	50	1.55
Nu-774	1-methyl-4-phenyl-4-butyroxy-piperidine	75	16	8.5	35	1.58
Nu-782	1-ethyl-4-phenyl-4-propionoxy-piperidine	75	18	2.2	50	1.55
Nu-801	1-ethyl-4-phenyl-4-butyroxy-piperidine	70	12	12	20	1.58
Nu-804	1-butyl-4-phenyl-4-butyroxy-piperidine	145	7	10	25	1.60
Nu-830	1-butyl-4-phenyl-4-propionoxy-piperidine	115	41	3.5	105	1.57
Nu-845	1-methyl-4-phenyl-4-acetoxy-piperidine	300	4	40	15	1.55
Nu-858	1-butyl-4-phenyl-4-acetoxy-piperidine	170	18	21	20	1.56
Nu-883	1-allyl-4-phenyl-4-propionoxy-piperidine	110	17	4.5	70	1.42
Nu-896	1-isopropyl-4-phenyl-4-propionoxy-piperidine	65	11	2.3	110	1.48
Nu-898	1-propyl-4-phenyl-4-propionoxy-piperidine	85	15	5.3	45	1.36
Nu-289	1-methyl-4-(p-methoxyphenyl)-1,2,5,6-tetra-hydropyridine	250	57	100	38	1.44
Nu-298	1-methyl-4-(p-dimethylcarbamoxyphenyl)-piperidine	600	10	150	40	1.25
Nu-353	1-methyl-2-(m-dimethylcarhamoxyphenyl)-piperidine	4.5	52	2.5	13	1.6
Nu-364	1-methyl-4-(m-dimethylcarbamoxyphenyl)-piperidine	175	13	150	19	1.38
Nu-366	1-ethyl-4-(m-dimethylcarhamoxyphenyl)-piperidine	600	4	300	18	1.35
Nu-370	1-ethyl-4-(p-methoxyphenyl)-1,2,5,6-tetrahydropyridine	400	54	200	29	1.55
Nu-541	1-methyl-4-(2',3'-dihydroxyphenyl)-4-carboxy-piperidine-γ-lactone	250	20	>100	10	1.11
Nu-758	1-methyl-4-(p-methoxyphenyl)-4-propionoxy-piperidine	400	7	>150	50	1.29
Nu-783	1-ethyl-4-phenyl-4 hydroxy-piperidine	>800	3	>200	10	1.03
Nu-802	1-ethyl-4-piperidone	>1000	3	>200	5	1.08
Nu-806	1-butyl-4-naphthyl-(1)-4-acetoxy-piperidine	650	8	>100	10	1.07

TABLE I—*Continued*

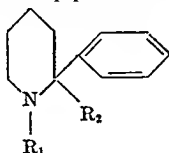| COMPOUND NUMBER	FORMULA	MOUSE SUBCUTANEOUS LD50	N	RAT ANALGESIC DOSE AD 1.40	N	ANALGESIC INDEX AT ABOUT ½ MOUSE LD50
		mgm./kgm.		mgm./kgm.		
Nu-811	1-butyl-4-naphthyl-(1)-4-propionoxy-piperidine	>1200	2	>200	10	1.11
Nu-813	1-methyl-4-phenyl-4 hydroxy-3-benzoyl-piperidine	600	15	>200	10	1.13
Nu-829	1-butyl-4-phenyl-4-hydroxy-piperidine	250	30	>200	10	1.09
Nu-840	1-butyl-4-benzyl-4-propionoxy-piperidine	360	17	>75	10	1.12
Nu-842	1-butyl-4-phenyl-4-fuoryloxy-piperidine	400	19	>150	15	1.17
Nu-848	1-ethyl-4-propionoxy-piperidine	>1000	4	>200	10	1.18
Nu-849	1-methyl-4-phenyl-4-benzoyloxy-piperidine	85	11	>50	5	1.09
Nu-853	1-butyl-4-phenyl-1,2,5,6-tetrahydropyridine	70	18	>50	10	1.23
Nu-854	1-butyl-4-phenyl-4-carbethoxyoxy-piperidine	350	10	>100	10	1.08
Nu-857	1-methyl-4-phenyl-4-β,β-dimethylacroyloxy-piperidine	200	8	>75	5	1.11
Nu-862	1-methyl-4-phenyl-4-propionoxy-3-benzoyl-piperidine			>100	5	1.17
Nu-863	1-methyl-4-phenyl-4-dimethylcarbamoxy-piperidine	500	3	>150	15	1.16
Nu-870	1-butyl-4-phenyl-4-(β-carbomethoxypropionoxy)-piperidine	>600	2	>100	5	1.08
Nu-871	1-butyl-4-phenyl-4-ethoxyacetoxy-piperidine	275	15	>125	25	1.22
Nu-880	1-ethyl-4-(p-methoxyphenyl)-4-propionoxy-piperidine	300	16	>100	10	1.25
Nu-881	1-butyl-4-(p-tolyl)-4-propionoxy-piperidine	195	19	100	10	1.42
Demerol	ethyl 1-methyl-4-phenylpiperidine-4-carboxylate	196	15	43.6	105	1.50
Morphine	Morphine sulfate	360	200	3.75	100	1.60
Codeine	Codeine phosphate	325	20	175	20	1.25

gesia the data would have to be treated by quantal response methods and many compounds could produce some but not complete analgesia. In all cases the analgesic indices given represent the maximum analgesia produced and as may be seen in figure 1 generally appeared one half hour after administration of the drug.

Complete analgesia actually means no reaction after indefinite exposure. However exposures of 15 seconds (with or without a reaction) almost invariably produced severe burns. We therefor stopped the tests at 10 seconds and arbitrarily considered this complete analgesia. In a few trials with some of the more potent compounds and morphine animals were exposed beyond 10 seconds and

it was noted that the majority of animals not reacting within 10 seconds would not react on further exposure. The reaction time of 10 seconds gives an index of 1.58 if the normal reaction time is just 4.0 seconds. All of the high indices in the tables include values representing complete analgesia in some or all rats tested. The index for complete analgesia ($\sqrt{10/t_1}$) was always averaged in as though it were a true graded analgesia.

Discussion of analgesic results. In Table I are listed the data on the 1-alkyl-4, 4-substituted piperidine compounds. Most of these are 4-acyloxy
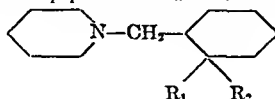
TABLE II

2-substituted piperidine compounds

COMPOUND NUMBER	FORMULA	MOUSE SUBCUTANEOUS LD50	N	RAT ANALGESIC DOSE AD 1.40	N	ANALGESIC INDEX AT ABOUT ⅙ MOUSE LD50
		mgm./kgm.		mgm./kgm.		
Nu-484	1-methyl-2-(2-dimethylcarbamoxy-6-naphthyl)-piperidine	600	21	>200	10	1.14
Nu-502	1-methyl-2-(2-methoxy-6-naphthyl)-piperidine	225	10	>100	15	1.10
Nu-527	1-methyl-2-(2-hydroxy-6-naphthyl)-piperidine	150	15	>50	15	1.13
Nu-810	1-methyl-2-phenyl-2-acetoxy-piperidine	400	20	>150	10	1.14
Nu 833	1-methyl-2-phenyl-2-propionoxy-piperidine	>800	2	>100	10	1.07
Nu-861	1-methyl-2-benzyl-2-propionoxy-piperidine	500	10	>150	10	1.10

compounds. Nu-353 is a 2-substituted compound but is included here because it is an isomer of Nu-366.

With one exception, Nu-353, the only compounds showing appreciable analgesic activity were those in the 1-alkyl-4-phenyl-4-acyloxy piperidine series, the first eleven compounds of table I.

Some other compounds listed were capable of producing an analgesic index as high as 1.20. This index was arbitrarily considered the dividing line between important and unimportant analgesia. It is possible that some of these or other compounds which possess low analgesic indices might be clinically useful in relieving some types of pain, as in arthritis for example. Nu-353 produced complete analgesia with a very low dose, but the material was exceedingly toxic. The high indices of 1.5 or over imply that one or more rats gave complete analgesia.
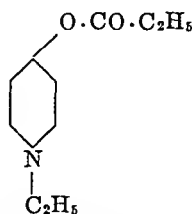
TABLE III

1-substituted piperidine and related compounds

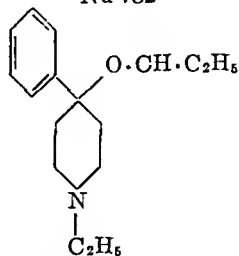| COMPOUND NUMBER	FORMULA	MOUSE SUBCUTANEOUS LD50	N	RAT ANALGESIC DOSE AD 1.40	N	ANALGESIC INDEX AT ABOUT ½ MOUSE LD50
		mgm./kgm		*mgm./kgm.*		
Nu-582	1-piperidinomethyl-2-cyclohexanone	425	17	>150	15	1.22
Nu-597	1-(4-morpholinylmethyl)-2-cyclohexanone	900	9	>200	10	1.08
Nu-622	2-(2-tetrahydroisoquinolylmethyl)-1-cyclohexanone	500	26	>150	10	1.08
Nu-636	2-piperidinomethyl-6-methoxy-tetralone-1	340	40	75	35	1.49
Nu-640	1-piperidinomethyl-2 hydroxy-2-(m-methoxyphenyl)-cyclohexane	490	15	>150	15	1.10
Nu-743	1-piperidinomethyl-2-propionoxy-cyclohexane	575	30	>150	10	1.05
Nu-756	1-piperidinomethyl-2 hydroxy-cyclohexane	450	11	>150	15	1.11
Nu-761	2-piperidinomethyl-6-methoxy-1-propionoxy-1,2,3,4-tetrahydronaphthalene	300	15	>50	10	1.12
Nu-776	1-piperidinomethyl-2-propionoxy-2-(m-methoxyphenyl)-cyclohexane	440	20	>200	10	1.05
Nu-786	2-piperidinomethyl-1-hydroxy-1,2,3,4-tetrahydronaphthalene	290	20	>100	10	1.11
Nu-805	2-piperidinomethyl-tetralone-1	170	15	>75	15	1.21

Certain conclusions are apparent in regard to the structural characteristics necessary to produce analgesia in this series:

(1) The 4-phenyl radical is essential, e.g.,
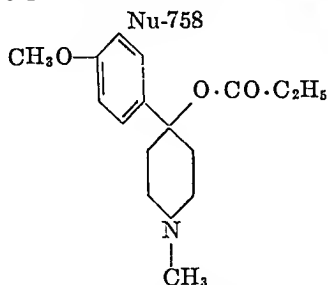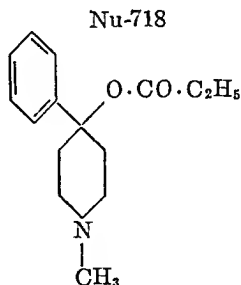
Nu-848

Nu-782

AD 1.40 > 200 mgm/kgm.
Highest index at any dose = 1.18

AD 1.40 = 2.2 mgmk./gm.

(2) The 4-phenyl radical may not be replaced by certain other substituents such as benzyl (Nu-840), naphthyl (Nu-806, Nu-811), p-tolyl (Nu-881) or p-methoxy-phenyl (Nu-758, Nu-880); e.g.,
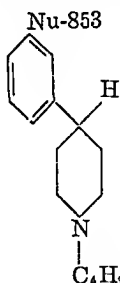
Nu-758

CH_3O— ... $O \cdot CO \cdot C_2H_5$ ... N—CH_3

AD 1.40 > 150 mgm./kgm.
Highest index at any dose = 1.29

Nu-718

$O \cdot CO \cdot C_2H_5$ ... N—CH_3
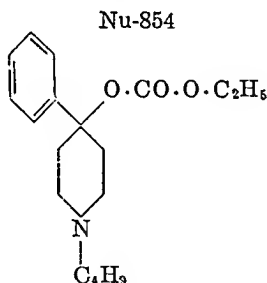
AD 1.40 = 1.4 mgm./kgm.

(3) The 4-acyloxy radical is essential since deviation from this structure reduces the activity, e.g.,

Nu-853

H ... N—C_4H_9

AD 1.40 > 50 mgm./kgm.
Highest index at any dose = 1.23

Nu-854

$O \cdot CO \cdot O \cdot C_2H_5$ ... N—C_4H_9

AD 1.40 > 100 mgm./kgm.
Highest index at any dose = 1.08
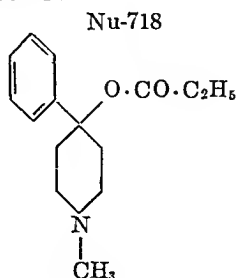
Nu-830

$O \cdot CO \cdot C_2H_5$ ... N—C_4H_9
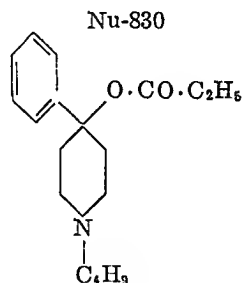
AD 1.40 = 3.5 mgm./kgm.

(4) Shifting the phenyl and acyloxy radicals from the 4- to the 2-position practically eliminates the analgesic activity, e.g.,
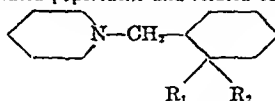
Nu-838

N—CH_3 ... $O \cdot CO \cdot C_2H_5$
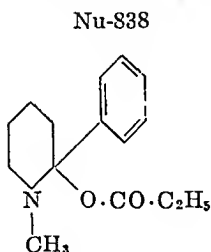
AD 1.40 > 100 mgm./kgm.
Highest index at any dose = 1.07

Nu-718

$O \cdot CO \cdot C_2H_5$ ... N—CH_3

AD 1.40 = 1.4 mgm./kgm.

TABLE III

1-substituted piperidine and related compounds

COMPOUND NUMBER	FORMULA	MOUSE SUBCUTANEOUS LD50	N	RAT ANALGESIC DOSE AD 1 40	N	ANALGESIC INDEX AT ABOUT ½ MOUSE LD50
		mgm /kgm		mgm /kgm		
Nu-582	1-piperidinomethyl-2-cyclohexanone	425	17	>150	15	1.22
Nu-597	1-(4-morpholinylmethyl)-2-cyclohexanone	900	9	>200	10	1.08
Nu-622	2-(2-tetrahydroisoquinolylmethyl)-1-cyclohexanone	500	26	>150	10	1.08
Nu-636	2-piperidinomethyl-6-methoxy-tetralone-1	340	40	75	35	1.49
Nu-640	1-piperidinomethyl-2 hydroxy-2-(m-methoxyphenyl)-cyclohexane	490	15	>150	15	1.10
Nu-743	1-piperidinomethyl-2-propionoxy-cyclohexane	575	30	>150	10	1.05
Nu-756	1-piperidinomethyl-2 hydroxy-cyclohexane	450	11	>150	15	1 11
Nu-761	2-piperidinomethyl-6-methoxy-1-propionoxy-1,2,3,4-tetrahydronaphthalene	300	15	>50	10	1.12
Nu-776	1-piperidinomethyl-2-propionoxy-2-(m-methoxyphenyl)-cyclohexane	440	20	>200	10	1.05
Nu-786	2-piperidinomethyl-1-hydroxy-1,2,3,4-tetrahydronaphthalene	290	20	>100	10	1.11
Nu-805	2-piperidinomethyl-tetralone-1	170	15	>75	15	1.21

Certain conclusions are apparent in regard to the structural characteristics necessary to produce analgesia in this series:
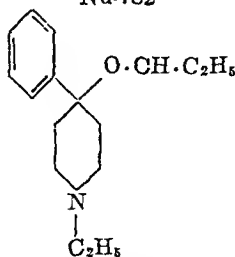
(1) The 4-phenyl radical is essential, e.g.,

Nu-848

Nu-782

AD 1.40 > 200 mgm/kgm.
Highest index at any dose = 1.18

AD 1.40 = 2.2 mgmk./gm.

In Table II there are no compounds which produced high analgesia.

In Table III Nu-582, 805 and 636 gave analgesic indices higher than 1.20. A high degree of analgesia was obtained with Nu-636 but only at a high dose. The relative safety of this compound is given in Table V and is 4.7, the same as for Demerol.

RELATIVE SAFETY. The first eleven compounds of Table I all exhibit a higher safety margin than Demerol (Table V). The most potent of these, Nu-718 and Nu-782 are seven and one half times as efficient as Demerol and about one-third as efficient as morphine. Although the whole series may be considered important it seems best to consider for extended investigation and clinical trial only the first few compounds. From present laboratory evidence preparations Nu-782, Nu-830, Nu-883 and Nu-896 appear the most desirable for further study. Nu-896 appears particularly desirable for further study. It has been found in this

TABLE IV

Fatal doses for mice and analgesic doses for rats for compounds 1 to 11

Figures represent milligrams per kilogram

1-ALKYL GROUP	4-ACYLOXY GROUP								
	-O CO CH₃			-O CO C₂H₅			-O CO C₃H₇		
	Compound number	LD50	AD 1.40	Compound number	LD50	AD 1 40	Compound number	LD50	AD 1.40
CH₃	Nu-845	300	40.0	Nu-718	40	1.4	Nu-774	75	8.5
C₂H₅				Nu-782	75	2.2	Nu-801	70	12.0
C₃H₇				Nu-898	85	5.3			
C₄H₉	Nu-858	170	21 0	Nu-830	115	3.5	Nu-804	145	10.0
CH₂—CH=CH₂				Nu-833	110	4.5			
CH(CH₃)₂				Nu-896	65	2.3			

series of compounds, that branching of the N-alkyl group inhibits the hydrolysis of the ester grouping which makes for stability of the aqueous solutions. Nu-718, the most active and possessing a high safety, unfortunately produces strong cataleptic symptoms in rats. While neither the mechanism nor the significance of these symptoms are clear it seems advisable to at least temporarily set aside this compound. Cataleptic symptoms produced by the other compounds are much less in evidence.

It is obvious that therapeutic efficiency based on toxicity in mice and analgesic activity in rats is not strictly valid. Nevertheless, it is justified for screening tests since mice are more easily available in large numbers than rats and are cheaper. Also the small quantities available of many of the compounds necessitated employing them sparingly.

An estimate of safety relative to morphine is somewhat fictitious just as is the therapeutic efficiency referred to above. The data can apply accurately only to similar experimental conditions. Because of other factors involved in considering the safety of a drug besides analgesic activity and lethal dose (as

(5) The carbon-oxygen linkage at the 4- position results in greater analgesia than with carbon-carbon linkage in this position, e.g.,

Demerol

CO·O·C₂H₅

N

CH₃

AD 1.40 = 43.6 mgm./kgm.

Nu-718

O·CO·C₂H₅

N

CH₃
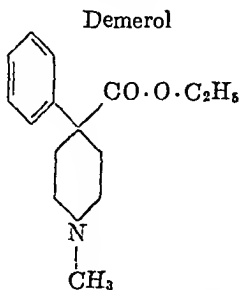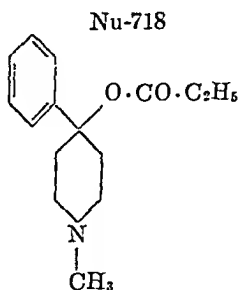
AD 1.40 = 1.4 mgm./kgm.

Among the 1-alkyl-4-phenyl-4-acyloxy compounds the number of carbon atoms in the two side chains influences the activity. This effect is summarized in Table IV. For the acyloxy group a maximum effect is reached with the three carbon atom chain of the propionoxy radical. With the 4-propionoxy group constant the single carbon atom alkyl group (methyl) on the nitrogen produces the maximum effect (Nu-718).

At a point when our study of these highly active compounds was well under way the work of Jensen and Lundquist (8) became known to us through an abstract (8a). Just recently their entire paper became available. They reported several 1-methyl-4-phenyl-4-hydroxypiperidine esters. Among these were five compounds of our own series, namely, Nu-783 (4-hydroxy), Nu-845 (4-acetoxy), Nu-718 (4-propionoxy), Nu-774 (4-butyroxy), and Nu-849 (4-benzoyloxy). E. Rekling and C. G. Wolffbrandt performed the biological tests for them. Their figures for toxicity in mice were closely similar to ours. They determined the smallest dose that would produce analgesia in mice as tested by a tail pinching method. They reported activities in terms relative to Demerol rather than in actual doses. They found the propionoxy compound (Nu-718) the most active. It was 5 to 10 times as active as Demerol while our data on rats indicated a 30-fold greater activity. The acetoxy derivative (Nu-845) we found equal to Demerol while their figure gave it half the activity. And the butyroxy compound (Nu-774) we found five times as active as Demerol while they simply indicated it to be less active. They reported a few higher esters which were all less active than Demerol thus substantiating the conclusion that the peak effect of the 4-acyloxy group resides in the propionoxy radical in this series. Jensen and Lundquist did not report any 1-alkyl compounds other than the 1-methyl.

In any one homologous series the analgesic activity and toxicity are approximately parallel. When the ratios of these effects (LD50/AD1.40) are arranged in descending order of their magnitude as in Table V it is found that the most potent compound (Nu-718) is not necessarily the most efficient.
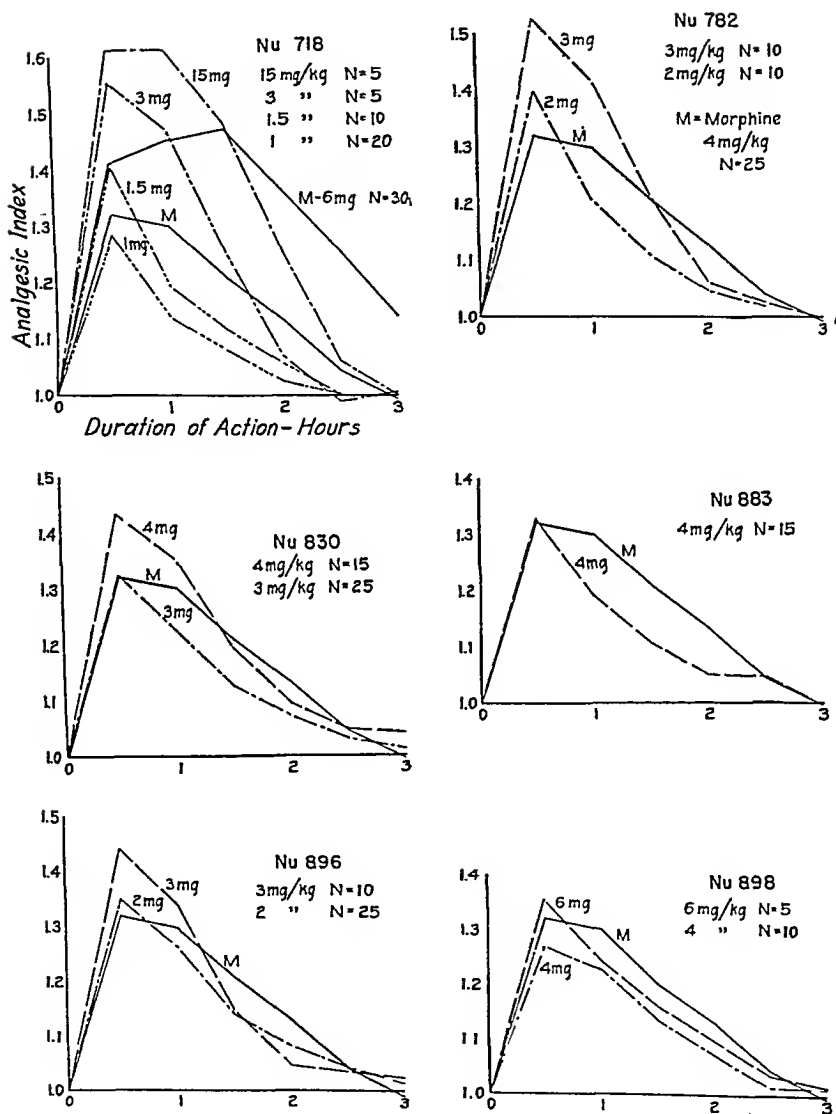
FIG. 1. ANALGESIC INDEX $(\sqrt{t_2/t_1})$ PLOTTED AGAINST TIME
Curves marked M were obtained with a 4 mgm./kgm. dose of morphine used in 25 rats.
The number of animals used with each compound is indicated on the charts.

respiratory depression and nausea and vomiting with morphine for example), and because different effects are submerged or magnified in different species, it seems probable that quite different relative safeties in man would be found. By these tests (Table V) Demerol is seen to be only one-twentieth as safe as morphine yet clinically it is at least as safe as morphine. These piperidine derivatives are as much as seven times safer than Demerol though only one-third as safe as morphine. How they will compare clinically remains to be demonstrated.

DURATION OF ACTION. Some of the data obtained have been plotted in figure 1 to show the duration of action. Time is given along the abscissae and the analgesic indices along the ordinates. Doses are listed along side each curve.

TABLE V

Relative safety of the more important compounds

COMPOUND	MOUSE LD50	RAT AD 1 40	RATIO LD50/AD 1 40	RELATIVE SAFETY MORPHINE = 100
	mgm /kgm	*mgm /kgm*		
Morphine	360	3 75	96	100
Codeine	325	175	1 85	1 9
Demerol	196	43 6	4 5	4.7
Nu-782	75	2.2	34	35
Nu-830	115	3 5	33	34
Nu-718	40	1 4	29	30
Nu-896	65	2 3	28	29
Nu-883	110	4 5	24	25
Nu 898	85	5 3	16	17
Nu-804	145	10	15	16
Nu-774	75	8 5	9	9
Nu-858	170	21	8	8
Nu-845	300	40	8	8
Nu 801	70	12	6	6
Nu-636	340	75	4 5	4 7
Nu-289	250	100	2 5	2.6
Nu-881	195	100	1 95	2

"M" stands for a 4 mgm./kgm. dose of morphine and is the same comparative curve in each chart. In addition a curve for a 6 mgm /kgm. dose of morphine is shown with Nu-718. The number of rats used is indicated on each chart.

These curves show that maximum analgesia is obtained at about one-half hour and then falls off more rapidly than morphine. Presumably these compounds are more rapidly destroyed or eliminated than morphine. This could be a factor involved in lower tolerance and may result in less likelihood of addiction though this does not necessarily follow It is to be noted that both metopon and desomorphine are shorter acting than morphine although the latter has a greater tendency to cause tolerance and addiction.

TOLERANCE. The question of tolerance has been investigated briefly. Three groups of 5 rats each were injected daily, 7 days a week, for 10 weeks. An
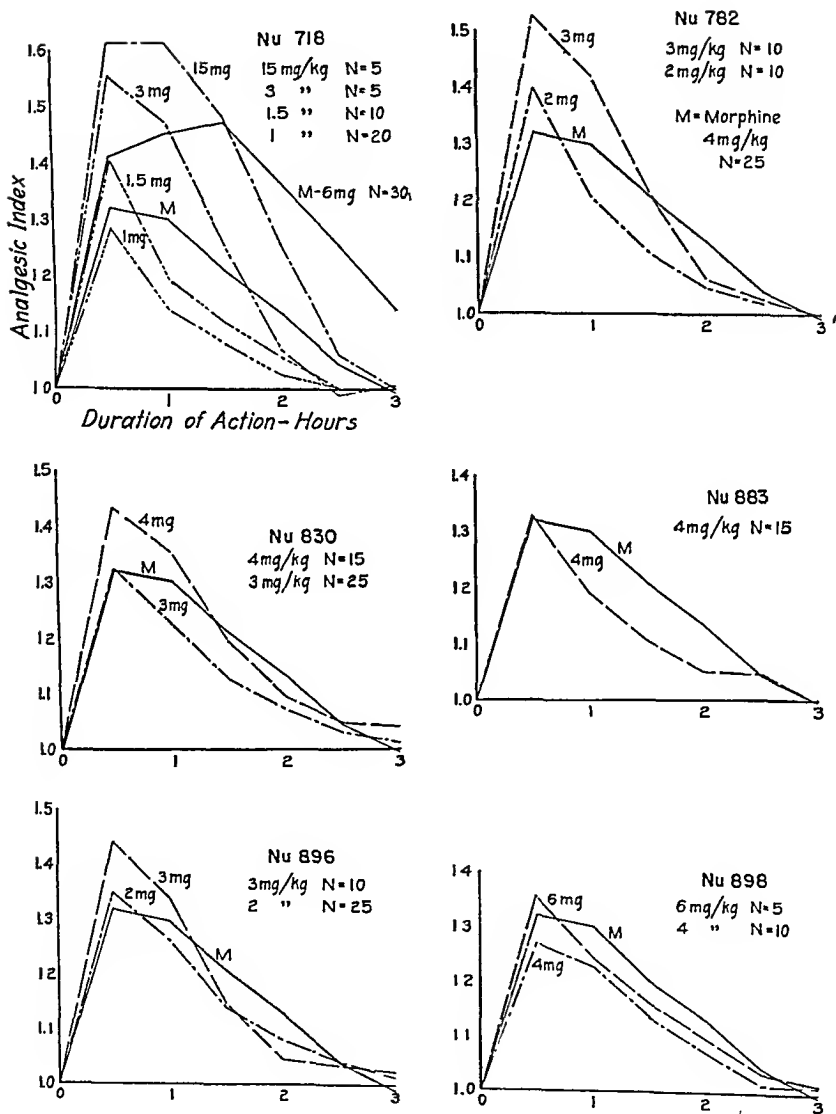
FIG. 1. ANALGESIC INDEX $(\sqrt{t_2/t_1})$ PLOTTED AGAINST TIME
Curves marked M were obtained with a 4 mgm./kgm. dose of morphine used in 25 rats.
The number of animals used with each compound is indicated on the charts.

analgesic test was made in the usual manner after the first injection and once a week thereafter. The data are plotted in figure 2.

The analgesic indices obtained on the first test were lower than expected since the doses employed were greater than the previously calculated AD 1.40 by 50% for morphine, 14% for Nu-830 and 30% for Nu-896. However, because of the wide scatter of values obtained for any given dose at different times on different groups of rats this is not too surprising.

The results indicate that tolerance although present does not develop as rapidly after these piperidine derivatives as after morphine. The analgesic effect of morphine steadily decreased until it almost completely disappeared. The analgesic effect of Nu-830 and Nu-896 decreased at a slower rate and then leveled off at an index of about 1.20.
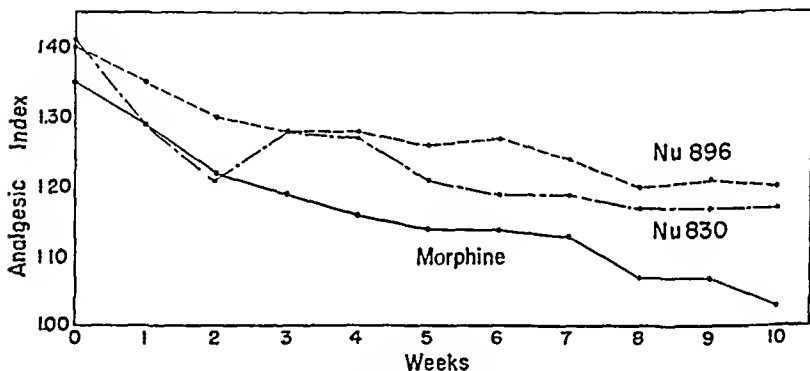

FIG. 2. TOLERANCE IN RATS

Daily injections of 6 mgm./kgm. of morphine, 4 mgm./kgm. of Nu-830 and 3 mgm./kgm. of Nu-896.

ANALGESIA IN CATS. As a matter of corroborating the results obtained on rats by the radiant heat method a few tests were made on cats using the tail-pressure method. We employed a weighted lever to produce pressure on the cat's tail rather than a spring device as did Eddy (9). The analgesic index was calculated thus:

$$\text{Analgesic index} = \sqrt{\frac{P_2}{P_1}}$$

where P_1 is the pressure eliciting a cry of pain in the untreated animal and P_2 the maximum pressure required after injection of the drug. Tests, usually repeated several times and averaged, were made at half-hour intervals. The method is not as satisfactorily in performance as the radiant heat method.

Nu-858 gave only a trace of analgesia although a moderate analgesia was anticipated at the dose employed. Nu-718, Nu-782, Nu-830 and Demerol showed high activity at doses comparable to those used in rats. Nu-774 pro-

duced good analgesia. Nu-743, negative on the rat test, was also negative in this test. The data are shown in Table VI.

SYMPTOMATOLOGY. The entire group of compounds tend to cause central stimulation in mice, rats and cats. As doses are increased, the signs of central stimulation become greater.

In mice toxic doses caused salivation, excitement, slight exophthalmos, convulsions, prostration and death. Fatal doses were not used in rats but moderately high doses caused excitement and salivation. Symptoms in cats are listed in Table VI.

TABLE VI

Analgesia and symptoms in cats

Doses in terms of hydrochloride salts administered subcutaneously. Analgesia determined by pressure on tail. Results are for individual animals.

COMPOUND NUMBER	DOSE	CONTROL TEST NO PRESSURE*	ANALGESIC INDEX	DILATATION OF PUPIL	EXCITEMENT	OTHER SYMPTOMS
	mgm/kgm					
Nu-858	10	7.7	1 08	++	0	
Nu-718	5	7.1	1 48	+++	++	Slight diarrhea
Nu-782	3	8.4	1.52	++	++	
Nu-782	3	6.9	1 42	++++	+	
Nu-830	3	5.1	1.25	++	0	Animal thin, sick feverish.
Nu-830	3	9 3	1.47	+++	++	Trace of rigidity
Nu-774	3	6.5	1 32	+++	0	
Nu-774	5	11.5	1.29	++	0	
Nu-743	50	9 9	0 99	++	+?	Pregnant. Delivery 4 hrs. after injection. Salivation
Demerol	15	5.8	1.63	++	+	Apprehensive but friendly
Demerol	50	8 5	1.80	++++	++++	Convulsion
Control		7.8	1 07	0	0	
Control		6.5	0.90	0	0	

* Pressure equals average of 4 to 7 separate tests.

+ to ++++ equal varying degrees of pupillary dilatation or excitement.

Of considerable interest was the appearance of catalepsy with a few of the compounds, particularly Nu-718. Catalepsy was produced in rats but not in mice. The compounds listed in Table VI did not produce catalepsy in cats in the doses employed. Bulbocapnine was used as a control but catalepsy failed to develop in the dose employed. A rat in the cataleptic state (with Nu-718) could be placed in any position and so remain for several minutes. One rat could be balanced vertically on its chin and forepaws and in this ludicrous position remain steady as long as 15 or 20 seconds. At any time during the cataleptic state all

rats when prodded could walk voluntarily across the floor for a few or many steps, although they appeared dazed.

SPASMOLYTIC ACTION. The compounds so far examined possess a fairly high degree of spasmolytic potency, both against acetylcholine and barium stimulated intestine. Data are given in Table VII in which comparisons are made with atropine, papaverine, Demerol and compound Ih (10). While those compounds tested stand up favorably against other spasmolytic agents, dose for dose, their spasmolytic activity is considered of secondary interest. Clinical doses giving good analgesia are not likely to produce appreciable spasmolytic activity but on the other hand, they would not produce the spastic effect of morphine.

TABLE VII

Spasmolytic equivalents

Figures represent concentration necessary to produce moderate and comparable relaxation

COMPOUND NUMBER	ACETYLCHOLINE CHLORIDE GUINEA PIG INTESTINE CONC 0 04 $\times 10^{-6}$	ACETYLCHOLINE CHLORIDE RABBIT INTESTINE CONC 0 2 $\times 10^{-6}$	BARIUM CHLORIDE RABBIT INTESTINE CONC. 200 $\times 10^{-6}$
Nu-782	6×10^{-6}	5×10^{-6}	40×10^{-6}
Nu-830	16	18	30
Nu-883	8	8	40
Nu-896	8	5	30
Demerol	6	5	20
Papaverine	5	10	10 (4)†
Compound Ih*	0.36		(5)
Atropine.	0.01		(15)

* 1-methyl-2,6-di(p-methoxyphenethyl)-piperidine ethane sulfonate (10).

† Figures in parentheses are for guinea pig intestine stimulated with same concentration

SUMMARY

1. A new series of piperidine derivatives has been examined for analgesic activity and other pharmacological actions.

2. Some of the compounds examined were found to produce analgesia in doses of $\frac{1}{3}$ to $\frac{1}{2}$ that of morphine, as measured in rats by a modified Hardy-Wolff technique.

3. The duration of analgesic action of the highly active compounds was slightly shorter than that of morphine.

4. Two of the highly active compounds were compared with morphine for the development of tolerance and were found to have considerably less effect in developing tolerance than morphine.

5. The relative safety of the highly active compounds proved to be $\frac{1}{3}$ that of morphine and 7 times that of Demerol when the criteria were fatal doses in mice and analgesia in rats.

6. Symptoms produced by successively increasing doses were those of central stimulation.

7. A few of the compounds could cause catalepsy in rats.

8. The compounds tested possessed high degree of spasmolytic action, both neurotropic and myotropic, but since the analgesic doses are so low this is likely to be unimportant.

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ABSORPTION AND RENAL EXCRETION OF THE TETRAETHYLAMMONIUM ION[1]

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The tetraethylammonium ion (TEA) has recently been shown to depress transmission in autonomic ganglia (1). The application of this drug in man as a diagnostic and therapeutic agent (2) has made it desirable to determine the manner in which the drug is eliminated.

Tetraethylammonium, in common with choline and other quaternary ammonium ions, possesses the property of forming a water insoluble, acetone soluble complex with Reinecke salt.[2] This reaction has been utilized in the quantitative determination of the drug in urine and in the intestinal contents.

METHODS. The analytical method follows in principle the procedure described by Jacobi et al. for the determination of choline (3). Since choline and other interfering nitrogenous compounds appear in the urine in only small amounts, direct analysis of the urine is possible. In plasma the level of TEA after effective doses probably does not exceed 1–2 mgm.%, a concentration too low to permit direct determination with this technique.

To a centrifuged sample of urine sufficient to contain between 0.5 and 7 mgm. of tetraethylammonium (calculated as the hydroxide) were added 3 cc. of a 2% solution of Reinecke salt in methanol. Precipitation was allowed to proceed under refrigeration for at least 4 and no more than 24 hours. Samples were filtered through sintered glass plates by suction, and the precipitates washed with three 2 cc. portions of 95% ethanol saturated with tetraethylammonium reineckate. The precipitate was then dissolved in acetone and carried through the filter into 15 cc. graduated centrifuge tubes. Acetone was added to a total volume of 10 cc. and the samples read in an Evelyn colorimeter, using a 520 μ filter. The red-violet to pink color of the reineckate follows Beer's law, the "K" value in our instrument being 7.49.

The identity of the tetraethylammonium reineckate was proved by molecular analysis of the precipitate recovered from dog urine.[3]

RESULTS

EXCRETION IN HUMAN SUBJECTS. Comparison was made of excretion rates after intravenous, intramuscular and oral administration of tetraethylammonium chloride or bromide. Urine was collected and the bladder washed at intervals through an in-lying catheter during the whole collection period in the subjects receiving the drug intravenously, and for the

first four hours in those injected intramuscularly. Subsequent specimens were voided voluntarily. Cumulative excretions, average and range, are listed in table 1.

After intravenous injection of 200 mgm. of TEA chloride in normal subjects the ion appeared in the bladder within 5 minutes. Excretion reached a maximum of 2 to 8 mgm. per minute within 10 to 15 minutes and diminished rapidly to less than 0.25 mg. per minute within 2 hours. Half of the administered dose was excreted in about 30 minutes, and about two-thirds in one hour (fig. 1). Such excretion rates are in the range of substances almost completely cleared from the renal arterial blood, and suggest that tubular excretion occurs in addition to glomerular filtration.

Three normal subjects received TEA chloride intravenously in a dose of 200 mgm., combined with 200 mgm. of sodium para-aminohippurate. In each case the cumulative excretion curve for TEA lay only slightly below that of PAH, again suggesting a tubular excretory mechanism for TEA.

After intramuscular administration of 10 to 20 mgm./kgm. excretion rates were much

TABLE 1

Cumulative percentage excretion of TEA Cl in human subjects

ROUTE	SUBJECTS	DOSE	5 MIN.	10 MIN.	20 MIN.	30 MIN.	60 MIN.
		mgm.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
I. V.	6 normal	200	4.0 (0–9)	16.0 (12–21)	38.0 (31–46)	51.0 (44–62)	68.0 (57–80)
I. V.	3 uremic	200	—	4.7 (2–8)	8.3 (4–13)	12.7 (5–18)	23.3 (13–30)
			30 MIN.	60 MIN.	2 HR.	4 HR.	8 HR.
I. M.	7 normal	20 mg./kg.	4.0 (1–13)	14.0 (1–37)	27.0 (7–60)	44.0 (21–75)	64.0 (50–88)
			2 HR.	4 HR.	8 HR.	16 HR.	24 HR.
Oral	5 normal	150 mg./kg.	3.0	5.1	7.1	8.5	10.0 (4–16)

more variable than after intravenous administration; the time of 50% excretion varied from 1½ to 8 hours in seven normal subjects (table 1, fig. 2).

Oral doses of 0.5 to 7.5 grams were given to seven subjects. At the end of 24 hours only 4 to 16% were recovered in the urine (table 1, fig. 2). Only minimal pharmacological effects were observed after the largest doses administered by this route.

Subjects with impaired renal function (as indicated by elevated N.P.N., low urea clearance, or diminished clearance of P.A.H.) excreted tetraethylammonium slowly. The time of 50% excretion of an intravenous dose may be delayed for hours (table 1).

EXCRETION IN DOGS. 1. *Continuous intravenous infusion.* Tetraethylammonium was administered by continuous intravenous infusion to 6 dogs under pentothal-barbital anesthesia. Priming doses of 0.5 to 4.2 mgm. (as the base) per kgm. were given; urine was collected from ureteral catheters. Although it was expected that the excretion rate would reach the infusion rate within one or two hours, the observed excretion rates did not exceed 74% of the infusion rate, even when the experiments were continued as long as 5 hours (table 2). The discrepancy does not represent TEA excreted by other channels or destroyed, for when urine collections were continued for several hours after cessation of infusion, as much as 95% of the total amount administered was recovered. Equilibrium

between blood and extracellular fluid might be expected much earlier than 5 hours. Since excretion became quite constant at about 60%–70% of the infusion rate within an hour
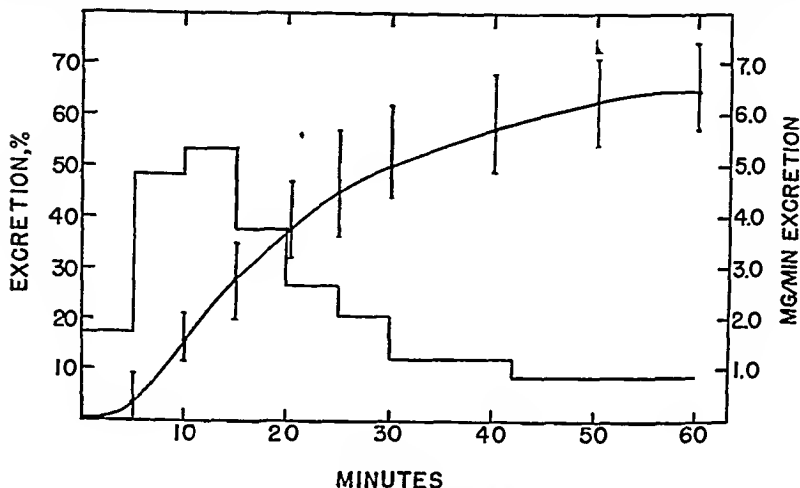

FIG. 1. Average excretion rate and average and range of cumulative excretion of TEA chloride after intravenous administration of 200 mgm. in 6 normal human subjects.
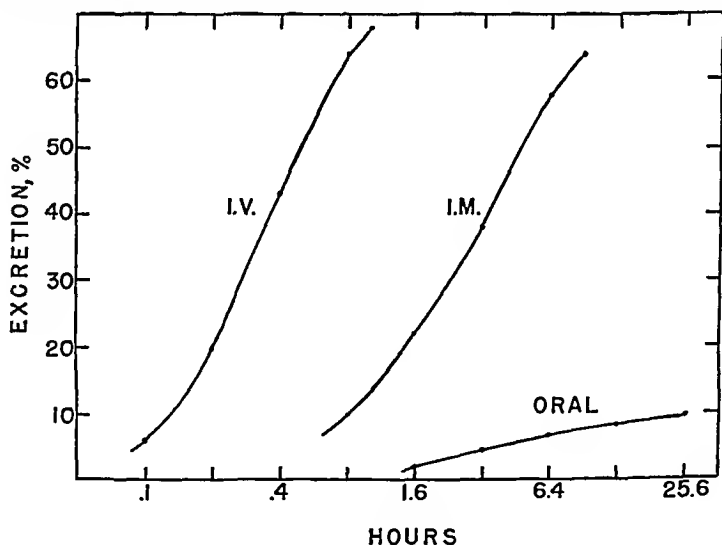

FIG. 2. Average cumulative excretion of TEA Cl after intravenous, intramuscular, and oral administration in normal human subjects.

after the beginning of infusion, it may be that the level of the drug in blood and extracellular fluid remains relatively constant, but that TEA slowly diffuses into tissue cells, or it may

be possible that the excretory mechanism becomes less efficient as the blood level increases. Since blood levels have not been determined this cannot be proved from the available data. Suspensions of saline-washed dog erythrocytes do not pick up significant amounts of TEA in 3 hours from solutions containing 100 times the probable blood level attained in the intact animal.

2. *Infusion into the renal artery.* In two experiments a solution containing TEA, sodium para-aminohippurate, and creatinine was infused through a needle inserted into the left renal artery. The excretion rate of the three substances was determined by analysis of urine collected from the left ureter, and corrected for accumulation in the blood by estimation of the excretion rate through the right kidney. In this way approximate simultaneous extraction ratios could be calculated for the three compounds. Results are listed in table

TABLE 2

Excretion rate in dogs during continuous infusion

EXP.	DOG WT.	PRIME*	INF. RATE (I.R.)	DURATION	MAX. EXCR. RATE (M.E.R.)	M.E.R./I.R.
	Kg.	*mgm./kg.*	*mgm./min.*	*hrs.*	*mgm./min.*	
3–9	8.7	2.5	.92	3.0	0.68	.74
3–20	9.7	2.5	.97	3.0	0.68	.70
2–23	9.5	0.5	1.0	1.5	0.60	.60
4–17	8.6	2.5	1.0	2.0	0.60	.60
4–6	13.0	2.7	1.56	5.0	1.07	.69
3–20	9.7	2.5	1.94	3.0	1.20	.62
1–18	9.5	4.2	8.25	4.5	5.10	.62

* Expressed as base.

TABLE 3

Simultaneous extraction of TEA, PAH, and creatinine

EXP.	TEA INFUSION RATE	CORRECTED EXCRETION	TEA EXTRACTION	PAH EXTRACTION	CREATININE EXTRACTION
	mgm./min.	*mgm./min.*	*per cent*	*per cent*	*per cent*
7–12	.50	.36	73	90.0	26
7–8	.78	.52	67	89.5	27
7–8	1.33	.77	58	89.0	30

3. It is evident that tubular excretion of TEA occurs; at the lowest infusion rate the extraction ratio reached 81% of the simultaneous value for PAH.

In five experiments the left kidney was perfused through tubing attached to a carotid or femoral artery of the same animal. The animals were heparinized, urine was collected separately from the two ureters, and TEA was infused at varying rates into the blood supply to the left kidney. Effective renal plasma flow and filtration rates for each kidney were determined by the usual methods, using PAH and creatinine and/or sodium thiosulfate.[4] Knowing the renal plasma flow and the TEA injection rate, it was possible to estimate the concentration of TEA in the plasma reaching the left kidney, and also to calculate the excretion rate as mgm. per 100 cc. renal plasma flow per minute. As the infusion continued

[4] Sodium para-aminohippurate was generously supplied by Sharp and Dohme, Inc., through the courtesy of Dr. Karl Beyer.

in these experiments, TEA appeared in increasing amounts in the urine of the right kidney. Values for the left kidney excretion, and the estimated blood levels, were corrected for this accumulation. The results obtained are presented in table 4. Figure 3 illustrates the relation of excretion rate and extraction ratio to plasma concentration as an average of the first three experiments in table 4.

In all experiments, the extraction ratio diminished as the calculated plasma level in-

TABLE 4

Excretion of TEA in dogs at increasing plasma concentrations

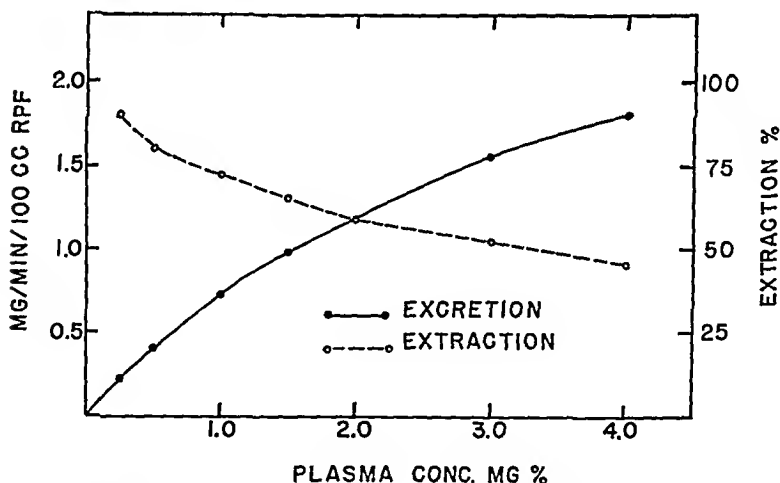| EXP.	PLASMA CONCENTRATION, MGM %						
	.25	.50	1 0	2 0	4 0	8 0	16 0
	Excretion, mgm /100 cc. RPF/min.						
5–13	.24	.45	.82	1.45	2.15	2.7	—
1–7	.20	.36	.64	.90	1.38	—	—
2–6	.23	.40	.70	1.20	1.95	2.4	—
1–14	—	.26	.46	.74	1.08	1.56	1.9
2–3	—	.33	—	—	—	—	—

FIG. 3. Average extraction ratio and excretion rate in relation to plasma concentration of TEA in 3 dog experiments.

creased. This would be expected since the tubular excretory mechanism must obviously have some upper limit. The extraction ratios approached the filtration fraction at plasma concentrations of 4 to 6 mgm.%, and in two experiments (1–14 and an additional experiment not included in table 4) the extraction ratio dropped below the filtration fraction at higher plasma concentrations. It is probable that when the concentration in the tubular urine becomes sufficiently high, reabsorption of the TEA ion, perhaps by simple diffusion, occurs in excess of the tubular excretory rate.

Lacking a satisfactory method for the determination of TEA in plasma, an attempt was

made in two experiments to estimate plasma concentrations indirectly. TEA was infused at a rate sufficient to produce effective ganglionic blockade as judged by blood pressure response. Renal plasma flow was determined by means of PAH clearance; the excretion rate of TEA was found to be from 0.7 to 1.2 mgm./100 cc. RPF/min. By reference to figure 3 it is seen that the plasma concentrations in these animals were in the range of 1.0 to 2.0 mgm.%.

3. *Intramuscular administration.* The duration of clinical effects following the intramuscular injection of TEA in human subjects is about 4 to 6 hours. On occasion it might be desirable to maintain a repository depot of the drug for long-sustained action.

Two preparations of TEA were used to test the possibility of delaying absorption from an intramuscular injection site. The first[5] contained TEA Cl, 50%, and beeswax, 10%, in propylene glycol; the second, a Pitkin's menstruum (4), contained TEA Cl 10%, gelatin 20%, glucose 10%, acetic acid 1%, and epinephrine 0.002%.
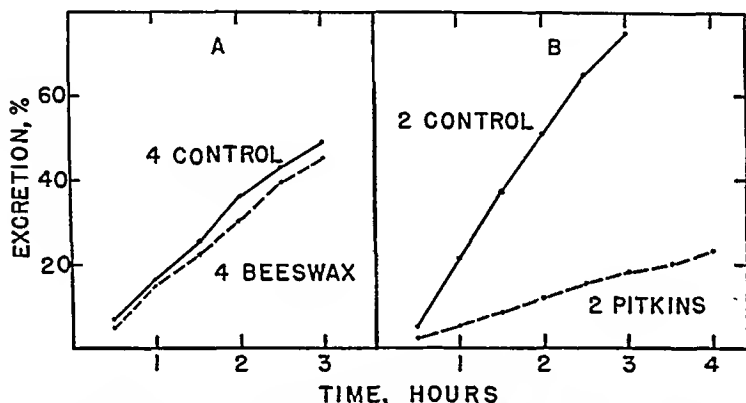

FIG. 4. Cumulative excretion of TEA after intramuscular administration in dogs. Control curves, TEA in aqueous solution; other curves as labeled.

Excretion rates were measured after administration of the beeswax mixture in a dose of 20 mgm./kgm. of TEA Cl in two dogs of equal weight. The average of two control excretion curves (TEA Cl in 10% or 50% aqueous solution) and two "beeswax" excretions in each animal are plotted in part A of figure 4. There was no significant difference in the excretion rate in the two groups. Since TEA is not oil-soluble, this result was perhaps to be expected.

Incorporation of TEA in Pitkin's medium considerably delayed excretion, as illustrated in part B of figure 4. The curves represent the average excretion rate, in two dogs, of aqueous TEA as compared with the Pitkin's mixture, each given in a dose of 20 mgm./kgm.

ABSORPTION FROM THE GUT. As shown above, only about 10% of orally administered TEA appears in the urine in man. Two possible explanations exist: either TEA is destroyed in traversing the portal circulation, or it is poorly absorbed. If hepatic destruction were significant, urine recoveries of 95% to 100%, after parenteral administration, would not be anticipated, for some of the injected drug must pass through the liver. Further to test the possibility of excretion or destruction by the liver, a comparison was made of the depressor action of TEA injected into the splenic and femoral veins. No difference other than that to be expected from delay and dilution in passing through the portal system was

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[5] Kindly supplied by Dr. George Rieveschl of Parke, Davis & Company.

evident. The liver does not destroy the drug, and poor absorption must account for the low excretion after the oral route of administration.

In three experiments the intestines were exposed in dogs under barbital anesthesia TEA in a concentration of 1:1000 in 0 9% NaCl was injected into washed loops of bowel in a volume sufficient to fill the segments. The loops were emptied and washed at the end of 1, 2, or 3 hours. Approximately 90% of the injected drug was recovered from the loops; the concentration in the bowel was increased by absorption of water.

It has been found (Gruhzit, 5) that a comparably low degree of absorption from the gut occurs in the mouse. The LD_{50} after oral administration of TEA chloride is approximately 14 times the intraperitoneal LD_{50}.

DISCUSSION. TEA is apparently quantitatively excreted by the kidneys in the dog and in man. The excretory mechanism is in part tubular; the extraction ratio is a function of plasma concentration, and the total amount excreted per minute is a function of both plasma concentration and renal plasma flow. At high plasma levels (probably considerably above those attained in the clinical use of the drug) tubular reabsorption may reduce the net excretion below the filtration level. Tubular reabsorption, if it represents simple diffusion, should depend on the concentration gradient attained as water is reabsorbed from the tubular urine, and should therefore depend upon the volume of urine elaborated. No attempt was made, however, to determine the effect of diuretic agents upon the rate of excretion of TEA.

The available data do not permit a definitive explanation of the discrepancy between excretion rate and infusion rate when TEA is administered at a constant rate to the intact dog. At an infusion rate of 1 mgm./min., an excretory plateau was reached at a level of 0.6 to 0.7 mgm./min., although the dog is capable of excreting the drug at much higher rates. It is apparent that the plasma concentration of free TEA must also reach a plateau; the cumulation of TEA must represent either binding of the ion in a non-filterable form, or diffusion into tissue cells. Assuming a similar mechanism in the human subject, cumulation should be demonstrable with frequent dosage, in spite of the rapid excretory mechanism.

Although the duration of clinical effects following a single intravenous injection is quite brief, it is doubtful whether any attempt should be made to prolong the action by intramuscular injection of a slowly-absorbed preparation. It is possible that the ordinary clinical intravenous dose would not endanger life even if never excreted, since equilibration with the extracellular fluid would reduce the plasma concentration below a dangerous level. But if a reservoir of the drug is deposited intramuscularly in a large dose, imparied renal function could lead to dangerously high plasma concentrations.

SUMMARY

1. TEA is quantitatively excreted by the kidneys in dogs and man after parenteral administration. In man 50% of an intravenous dose appears in the urine in 30 minutes, and 50% of an intramuscular dose in about 4 hours.

2. No delay in absorption from an intramuscular injection site was caused by incorporating the drug in a propylene glycol-beeswax mixture, but absorption was delayed after injection of the drug in Pitkin's menstruum.

3. The total excretion after oral administration in man is only 4 to 16% of the dose given. It is probable that poor absorption, rather than enteric or hepatic destruction, accounts for the low recovery.

4. The speed of excretion after intravenous injection in man, and the high extraction ratio obtained in the dog when the drug is infused into the renal artery, indicate that tubular excretion occurs in addition to glomerular filtration.

5. Since at high blood levels the extraction ratio falls below the glomerular filtration fraction, tubular reabsorption may also occur.

6. The drug should be used with caution in patients with severe renal damage.

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THE INFLUENCE OF VARIOUS SUBSTANCES ON CHRONIC SELENIUM POISONING[1]

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The toxicity of selenium has been recognized before its relation to public health problems and livestock poisoning were fully appreciated. Inasmuch as selenium contaminates grains and vegetables consumed by human beings as well as livestock the factor or factors which influence selenium poisoning merits consideration.

Limited number of investigations reported in the literature indicate that toxicity of selenium may be influenced by various factors. Smith (1) observed the protective action of high protein diets against organic and inorganic selenium poisoning in rats. Gortner (2) studied the effects of various proteins such as casein, lactalbumin, gelatin, and edestin against chronic selenium poisoning produced by inorganic selenium. He obtained good protection with 30 per cent casein; lactalbumin counteracted some of the toxic effects of selenium, but edestin and gelatin exerted no detoxifying action. Moxon and DuBois (3) reported increased mortality with selenium from seleniferous wheat when 5 p.p.m. of each of the following; fluorine, molybdenum, chromium, vanadium cadmium, nickel or uranium was added to the drinking water. Sodium tungstate in the same amount prevented to some extent the typical liver damage, and sodium arsenite completely prevented the symptoms of selenium poisoning in rats. Rosenfeld and Beath (4) reported the beneficial effects of high and medium protein diets on chronic selenium poisoning of sheep due to inorganic selenium.

These studies indicate that dietary factors can reduce and the addition of other chemicals can enhance or decrease the toxicity of selenium.

Since chronic selenium poisoning is an economic as well as a public health problem, we attempted to investigate what factors influence selenium intoxication.

In the present experiments we have studied the influence of ascorbic acid, beet pectin and potassium iodide on chronic selenate and selenite poisoning.

We have observed that in chronic selenosis the ascorbic acid of the blood and liver dropped to a very low level (5). Since ascorbic acid can reduce the selenite compounds to elemental selenium *in vitro*, in which form selenium is harmless (6), we thought it would be of some value to investigate the action of increased ascorbic acid intake on chronic selenosis.

Reports on the effect of pectin on heavy metal poisoning indicate that pectin reduced the toxicity of these compounds by forming an insoluble salt (7). *In*

vitro, selenium forms an insoluble salt with pectin (8), and this suggested the *in vivo* study of the effect of pectin in chronic poisoning.

The use of potassium iodide as an aid in the excretion of lead has been recognized (9); therefore, we have included this compound in this study. By these factors we aimed to influence selenium in the following manner: (a) reduce it to a non-toxic form; (b) form an insoluble salt; (c) increase the excretion of selenium.

METHODS AND MATERIAL. Adult male and female Sprague-Dawley rats were used in these experiments, and they were fed ad lib. with Purina Laboratory Chow. The weights of the animals varied from 200 to 250 grams at the beginning of the experiment. The animals were divided into groups of five or ten rats. The weights in the groups were balanced so that each varied only ± 20 grams. Crystalline ascorbic acid was dissolved in saline just before use, and it was given by stomach tube in amounts indicated in the experiments. The sodium salts of selenate and selenite were dissolved in saline, and the amount of selenium present was determined according the method of Kline (10). In experiment I each rat received 0.15 mg. of selenium per day, and in experiments II and III 1.5 mg. of selenium per kg. was given to each animal daily. Two per cent purified beet pectin was prepared according the method reported by Roboz and Van Hook (11).² One cc. of two per cent pectin was given to each animal daily. The selenium, ascorbic acid and pectin were administered by stomach tube to all the animals. Potassium iodide was dissolved in saline, 28 mg. per rat was given daily by intraperitoneal injection, and the selenium was administered by the same route.

The ascorbic acid, pectin and potassium iodide were given from 30 to 60 minutes before the selenium.

Groups of five rats received pectin, ascorbic acid and potassium iodide in the same amounts and by the same route as the experimental groups but received no selenium; two groups of five animals served as controls for the selenate and selenite compounds.

RESULTS. 1. *Action of ascorbic acid and potassium iodide on chronic selenosis* The effects of ascorbic acid in chronic sodium selenite poisoning were variable, as can be seen from Table I, since rats receiving 10 and 20 mg. of ascorbic acid lived longer than those receiving 200 mg. This suggested that the action of ascorbic acid *in vivo* was not similar to that observed *in vitro*. If this relationship existed *in vivo,* then a quantitative detoxification would have been present with a constant amount of selenium. However, this was not observed in these groups of animals. Several rats from the various groups lived at the termination of the experiment of 60 days, at which time selenium feed'ng was discontinued. These animals appeared normal for two months, then suddenly developed edema of the extremities and ascites (fig. 1). This indicates that lesions once formed, due to the ingestion of selenium, remain permanent even after the ingestion of the toxicant is discontinued. Beath (12) reported that months after grazing upon seleniferous plants, animals showing no signs of poisoning, suddenly developed toxic symptoms and died in from 1 to 6 days.

At the time of death the animals were either cachectic or showed ascites and edema in the tissues. The ascitic fluid sometimes appeared blood-tinged or was pale yellow in color. The development of ascites was not prevented by the

² We are indebted to E. Roboz, N.R.R I., University of Wyoming for supplying the pectin.

administration of ascorbic acid but it appeared to be related to liver injury. The amount of fluid in the abdominal cavity on the average varied from 43 grams to 102 grams per rat in the various groups.

In experiment II we gave a constant amount, 100 mg., of ascorbic acid or 28 mg., of potassium iodide, in addition to the 1.5 mg. selenite and selenate selenium per kg. per rat, per day. Table II shows the results obtained. It is evident that if we increased the selenium intake, the duration of life decreased, as can be

TABLE I

*Effect of varying amounts of ascorbic acid on chronic selenosis**

AMOUNTS OF ASCORBIC ACID GIVEN DAILY	SURVIVAL PERIOD†	NO. DEVELOPED ASCITES	AMOUNT OF FLUID/RAT†
mg.	*days*		*grams*
10	49	5	43
20	53 (3)	4	78
50	47 (2)	4	102
100	51 (2)	5	84
200	46 (3)	3	78
0	45	3	80
200‡	All living and well	0	0

*̤0.15 mg. of sodium selenite selenium was administered daily to each rat.
† Average of the results of 5 rats.
‡ Control for ascorbic acid. No selenium.
( ) Indicates the number of rats which developed latent edema and ascites two months after selenium feeding was discontinued.

FIG. 1. ASCITES AND EDEMA DEVELOPED TWO MONTHS AFTER SELENIUM AND ASCORBIC ACID ADMINISTRATION WAS DISCONTINUED

seen by comparing the results in Tables I and II. Whether we varied the selenium or the ascorbic acid intake, the average duration of life was affected very little when compared with the controls. There was some increase in the duration of life, but this difference was not considered significant. The development of edema, ascites, or cachexia was not prevented by ascorbic acid in the selenate or selenite groups. The cachetic animals lost from 35 to 50 per cent of their original weights. The tabulation of weights would be of little value for purposes of comparison because all animals lost weight at the beginning of the experiment, and then there was a gradual increase in the weights with the even-

tual development of a definite ascites. The factor or factors which produce cachexia in one animal and ascites in others under identical experimental conditions must be due to the inherent difference in the experimental animals.

At autopsy the organs grossly showed various grades of damage, and in a few cases the changes were similar to those observed in chronic selenium poisoning

TABLE II

Effect of constant amount of ascorbic acid and KI on selenite and selenate poisoning

FORM AND AMOUNT OF SELENIUM	NO. OF RATS	AMOUNT OF SUBSTANCE GIVEN PER RAT	SURVIVAL PERIOD	NO. SHOWED ASCITES	AMOUNT OF FLUID/RATS
			days		grams
1.5 mg. of Se per kg. as sodium selenate	9	100 mg. ascorbic acid	49	7	119
	5	28 mg. KI	35	3	47
	10	0	40	6	65
1.5 mg. Se per kg. as sodium selenite	9	100 mg. ascorbic acid	44	5	102
	5	28 mg. KI	23	4	50
	10	0	35	6	80
0	5	100 mg. ascorbic acid	All normal	0	0
0	5	28 mg. KI	All normal	0	0

FIG. 2. ELONGATED AND CURVED NAILS DEVELOPED IN RATS WHICH RECEIVED SODIUM SELENATE AND ASCORBIC ACID

in cattle (13). With the administration of selenate and ascorbic acid several of the rats showed hemorrhages around the nail and elongated nails (fig. 2) which resembled the elongation of the hoofs in cattle and horses in "alkali disease". Whether this peculiar manifestation of chronic selenosis is associated with the combination of selenate and ascorbic acid, we cannot state definitely.

No similar observations in rats have been reported by previous investigators. Most workers emphasized the fact that the elongation of nails in experimental animals does not occur when inorganic selenium or seleniferous grains are used as toxicants (14).

The administration of potassium iodide decreased the life span of the animals as can be seen from Table II. The decrease in the duration of life was most marked in the selenite group. The development of intoxication was similar to that observed in chronic poisoning, but the course of the disease was more rapid. These results indicate that the potassium iodide enhances the toxicity of selenium.

2. *The action of beet pectin on chronic selenosis.* Table III gives the results obtained by the administration of beet pectin and selenate and selenite selenium.

TABLE III

Effect of 2% beet pectin on chronic selenite and selenate poisoning

FORM AND AMOUNT OF SELENIUM	NO OF ANIMALS	2% PECTIN DAILY	SURVIVAL PERIOD OF THE GROUP	NO SHOWED ASCITES	FLUID IN ABDOMINAL CAVITY/RAT
		cc.	days		grams
1.5 mg. per kg sodium selenate selenium daily	10	1	61	1 (1)	113
	5	0	39	4	65
1.5 mg per kg sodium selenite selenium daily	10	1	72	(4)	
	5	0	32	3	79
0	5	1	well and alive	0	0

( ) Indicate the number of animals which are alive 5 months after the beginning of the experiment and ascites and edema started to develop.

Although pectin did not give complete protection, it lengthened the lives of the animals. The average duration of life with selenate selenium and pectin was 61 days while the controls lived only 39 days. With the selenite selenium, an increase of life from 32 days to 72 days was observed. Since selenite is more toxic than selenate, the survival of four animals and the increased protection afforded by the pectin in this group suggest that pectin either combined with the selenite or prevented the absorption of selenium and thereby reduced its toxic effects. The mechanism whereby pectin reduced the toxicity of selenium is being investigated in our laboratory and will be the subject of another report.

The daily administration of pectin prevented the development of ascites in all but one animal during the experiment (80 days). Animals which were living at the termination of the experiment, however, gradually developed ascites two months after selenium and pectin administration was discontinued. Therefore, the beet pectin did not give complete protection against liver damage, and ascites developed due to portal obstruction

At autopsy the livers and other organs grossly did not show the pathological changes of chronic selenium poisoning in the pectin groups. There was no cirrhosis present in those animals which died during the experimental period, but the livers showed congestion, hemorrhages, and other pathological changes which resembled acute or sub-acute types of selenium poisoning.

SUMMARY

Various amounts of ascorbic acid given *per os* to rats receiving a constant amount of sodium selenite (0.15 mg. selenium) daily did not protect the organs from injury nor prevent the development of ascites. Duration of life in the different groups showed considerable variations and there was no quantitative relation between the amount of ascorbic acid and the survival period. Rats receiving 10 and 20 mgs. of ascorbic acid lived longer than those receiving 200 mg.

The daily administration of a constant amount (100 mg.) of ascorbic acid gave no protection against an increased amount (1.5 mg. per kg. of body weight of selenium) of sodium selenate and selenite. The rats showed pathological changes similar to those observed in chronic selenium poisoning in cattle.

The intraperitoneal injection of 28 mg. of potassium iodide daily increased the toxicity of selenate and selenite selenium.

The daily oral administration of beet pectin increased the life of the animals and prevented the development of ascites during the experiment. Ascites, due to portal obstruction, developed two months after selenium feeding was discontinued.

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THE TOXICITY AND PHARMACOLOGICAL ACTION OF THE NITROGEN MUSTARDS AND CERTAIN RELATED COMPOUNDS[1]

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The resumption of research in chemical warfare at the advent of World War II directed attention to a new class of vesicants, the nitrogen mustards. All of the nitrogen mustards contain two or more β-chloroethyl groups and thus resemble mustard gas (*bis(β chloroethyl)sulfide*), the atom linking the β-chloroethyl groups being nitrogen instead of sulfur.

The present paper reports the toxicity and gross pharmacological action of certain of these compounds when administered to small animals by subcutaneous, intraperitoneal or intravenous injection. A number of closely related compounds and reaction products are included. These data are primarily of value for the light they throw on the mechanism of action, and especially the relation of chemical constitution to the production of leucopenia.

The leucopenic action of the nitrogen mustards was first reported for HN2 by Lushbaugh (1). The present studies, with those reported elsewhere (2) on gross and micropathology, and the studies of Gilman and others in man (3) reveal that this leucopenic action is one of the important properties of this group of compounds.

The chief compounds studied here were *bis(β-chloroethyl)ethylamine* (HN1), *bis(β-chloroethyl)methylamine* (HN2), *tris(β-chloroethyl)amine* (HN3), and *bis(β-chloroethyl)isopropylamine*.

EXPERIMENTAL. The amines listed in tables 1 and 3 were administered intravenously, subcutaneously or intraperitoneally as solutions of their respective hydrochlorides in 0.9 per cent saline. The compounds in table 2 and those in table 3 other than those named above were administered as solutions of the indicated salt. The concentration of the solutions was such that 0.1 cc. of the solution contained the dose for a 20 gm. mouse when injected intravenously, subcutaneously or intraperitoneally; 0.5 cc. contained the dose for a 200 gm. rat injected subcutaneously; 0.1 cc. contained the dose for a 200 gm. rat injected intravenously, and 1.0 cc. contained the dose per kilogram of body weight for rabbits and dogs. Intravenous injections were made in a tail vein of mice, the femoral vein of rats, a marginal ear vein of rabbits and the jugular vein of dogs. Subcutaneous injections were made in the flank.

The free amine was employed for cutaneous application. The amine was prepared by displacement from an ice-cold solution of the hydrochloride by the addition of ice-cold N

NaOH. The oily amine was separated from the aqueous layer, and the latter extracted three times with separate portions of chloroform. The chloroform extracts and the amine layer were combined, washed with cold water and dried over anhydrous Na_2SO_4. The dry solution was evaporated under water pump pressure at temperatures between 50 and 60°C. The free amines were applied by means of a calibrated drod to the skin of animals securely restrained. Free evaporation of the agent was permitted and at the end of two hours the residual agent was removed by scrubbing the area of application three times with a cotton swab soaked in ether. Animals were confined singly in cages, permitted food and water *ad libitum* and observed for 15 days. To determine the LD_{50}, the logarithm of the dose was plotted against the per cent mortality on a probability scale. A straight line was drawn free-hand to fit the points and the dose at which this line crossed the 50 per cent ordinate was taken as the LD_{50}.

TABLE 1

The toxicity of the nitrogen mustards for various species $(LD_{50}$ *in mg./kg.)*

COMPOUND	ROUTE ANIMAL	CUTANEOUS	SUBCUTAN-EOUS	INTRAVENOUS	ORAL
Ethyl-*bis*(β-chloroethyl)amine (HN1)	Mouse Rat Rabbit	13 17 *ca.* 15	1.2 1.0	0.5 *ca.* 2.0	
Methyl-*bis*(β-chloroethyl)amine (HN2)	Mouse Rat Rabbit Dog	29 22 *ca.* 15	2.6 1.9	*ca.* 2.0 1.1 *ca.* 1.6 1.0	20* 10†
Tris(β-chloroethyl)amine (HN3)	Mouse Rat Rabbit Dog	7 4.9 19 1.0	2.0	0.7 2.5	
Isopropyl *bis* (β-chloroethyl)-amine	Mouse Rat Rabbit		1.1	0.5 *ca.* 2.0	22.0

* Fed mice.

† Fasted mice.

RESULTS. The LD_{50} for the nitrogen mustards for various species by different routes are shown in table 1. Applied to the skin HN1, HN2 and HN3 in mice and rabbits and HN3 in rats are more toxic than H. In addition, the nitrogen mustards are more toxic than H when injected subcutaneously, a circumstance which is possibly related to the difference in chemical properties (*vide infra*). Other LD_{50} values in table 1 are comparable to those for H. The values for oral administration of HN2 in mice indicate that the presence of food in the gastro-intestinal tract exerts a protecting influence. This circumstance probably arises from the reaction of HN2 with constituents of the diet or their digestive products.

Therapy. Attempts to alter the LD_{50} by the prophylactic or therapeutic administration of various agents have been unsuccessful. The incorporation of one

or 2 per cent sulfapyridine in the diet of mice intoxicated subcutaneously by 4 mg./kg. of HN2 did not protect the hematopoietic organs, prevent weight loss or leucopenia nor alter ultimate mortality, although it prolonged average survival time from 91 to 123 hours.   Parenteral administration of 10 mg. of sulfapyridine

TABLE 2

The toxicity of derivatives of the nitrogen mustards (LD_{50} in mg./kg.)

COMPOUND	REMARKS	ROUTE	ANIMAL	LD_{50}
I. Ethyl - β - chloroethyl -ethylenimonium picrylsulfonate		subcutaneous intravenous intravenous	Mouse Rat Rabbit	2.0 *ca.* 0.5 *ca.* 3.0
II. Ethyl - β - chloroethyl- β - hydroxyethyl picrylsulfonate		subcutaneous intravenous intravenous	Mouse Mouse Rabbit	8.0 8.0 5–10
III. Ethyl - β - hydroxy- ethyl - ethylenimon- ium picrylsulfonate		subcutaneous intravenous intravenous	Mouse Mouse Rabbit	*ca.* 5.5 *ca.* 5.0 5–6
IV. Methyl - β - chloro- ethyl - ethylenimon- ium picrylsulfonate		subcutaneous intravenous	Mouse Mouse	2.4 *ca.* 1.5
V. Methyl - β - chloro- ethyl - β - hydroxy- ethylamine hydro- chloride		subcutaneous intravenous intravenous intraperitoneal	Mouse Mouse Rabbit Mouse	16 22.5 *ca.* 12 34.0
VI. Methyl-β-acetoxy- ethyl-β-chloroethyl- amine	Dissolved in PG	intravenous	Mouse	*ca.* 36
VII. Methyl-β-hydroxy- ethyl-ethylenimon- ium picrysulfonate		intravenous intravenous intraperitoneal	Mouse Rabbit Mouse	4.2 3–5 7.5
Bunte salt of methyl-*bis*(β- chloroethyl)-amine		subcutaneous intravenous intravenous	Mouse Mouse Rabbit	500 200 50
N,N'-dimethyl-N,N'-*bis*(β- chloroethyl)-piperazinium dichloride		subcutaneous	Mouse	*ca.* 500

intraperitoneally in mice intoxicated subcutaneously with HN2 gave results simi- lar to those obtaining in mice fed sulfapyridine.   Rats intoxicated intravenously with 3 mg./kg. of HN2 hydrochloride were similarly unaffected by the daily administration by stomach tube of 125 mg. of sulfapyridine.   Medication with

TABLE 3

The toxicity of compounds related to the nitrogen mustards (LD_{50} in mg./kg.)

COMPOUND	ANIMAL	ROUTE	REMARKS	LD_{50}
I. Compounds containing one β-chloroalkyl group per N				
A. Secondary amines				
VIII. 1. Methyl-β-chloro-ethylamine	Mouse	intravenous	Non-leucotoxic	*ca.* 100
IX. 2. Ethyl-β-chloro-ethylamine	Mouse	intravenous	Non-leucotoxic	*ca.* 100
B. Tertiary amines				
X. 1. Diethyl-β-chloro-ethylamine	Mouse	intravenous		100
	Mouse	subcutaneous	Non-leucotoxic	100
	Rabbit	intravenous		40–100
II. Compounds containing two β-chloroalkyl groups per N				
A. Secondary amines				
XI. 1. *Bis*(β-chloroethyl)-amine	Mouse	subcutaneous	Leucotoxic at LD_{50}	20–33
B. Tertiary amines				
XII. 1. Allyl-*bis*(β-chloro-ethyl)amine	Mouse	subcutaneous	Leucotoxic at LD_{50}	4–6
XIII. 2. Vinyl-β-*bis*(β-chloro-ethyl) amino-ethyl sulfone	Mouse	subcutaneous		9.0
	Rabbit	intravenous	Mildly leucotoxic	2.55
XIV. 3. N,N,N'N'-*tetrakis*-(β-chloroethyl)-ethylenediamine dihydrochloride	Mouse	subcutaneous		*ca.* 26
	Mouse	intravenous		*ca.* 7.5
	Rat	subcutaneous	Leucotoxic	*ca.* 19
	Rat	intravenous		*ca.* 3.8
	Rabbit	intravenous		*ca.* 2.5
C. Quaternary salts				
XV. 1. Dimethyl-*bis*(β-chloroethyl) am-monium chloride	Mouse	subcutaneous	Non-leucotoxic	100–200
XVI. 2. *Bis*(β-chloroethyl)-morpholinium chloride	Mouse	subcutaneous	Non-leucotoxic	100
D. Unclassified				
1. *Bis*(β-chloroethyl)-chloroamine	Mouse	intravenous	Impure preparation,	50
	Mouse	subcutaneous	Non-leucotoxic	360
2. *Bis*(β-chloroethyl)-nitrosoamine	Mouse	subcutaneous	Non-leucotoxic	100–200
3. *Bis*(β-chloroethyl)-formamide	Mouse	intravenous	Non-leucotoxic	300–500

atropine or prostigmine and therapeutic treatment with saline, glucose or lactate were without effect on survival time of rats intoxicated intravenously with 2.5 mg./kg. of NH2. Atropine had no effect in preventing convulsions induced

in mice by supra-LD$_{50}$ doses of HN3. Massive doses of desoxycorticosterone acetate (50 mg./kg. daily) given subcutaneously had no effect on the survival time of mice intoxicated subcutaneously with 4 mg./kg. of HN2 hydrochloride. Similarly, tolerated doses (*ca.* 875 mg./kg.) of pentnucleotide administered subcutaneously before and after, 0.5 cc. of egg white administered intraperitoneally before and after, or biotin administered before and after LD$_{50}$ doses of HN3 were without effect on the mortality of mice intoxicated subcutaneously. The administration (50 mg./kg. dissolved in PG) of BAL (2,3-dimercaptopropanol) intraperitoneally to rats 6 hours after the cutaneous application of a 1.5 LD$_{50}$ dose of HN3 decreased the survival time. Extended medication with aminopyrine (10 mg./kg. given twice daily by stomach tube) had no influence on the mortality or survival time of mice injected subcutaneously with an LD$_{50}$ and 2 LD$_{50}$ doses of HN3. Large doses (0.5 to 7.5 rat units) of adrenal cortical hormone given intraperitoneally twice daily to rats failed to influence survival time after intravenous intoxication with LD$_{50}$ doses of HN3.

Pharmacologic properties of the nitrogen mustards. *Bis(β-chloroethyl) ethylamine:* In mice 50 to 250 mg. of HN1 injected intravenously per kg. of body weight caused violent continuous convulsions terminating fatally in one to seven minutes, while 25 mg./kg. were fatal in 20 to 60 minutes. Injected subcutaneously in mice 100 to 200 mg./kg. caused convulsions and tremors and were fatal in 15 to 30 minutes, and 25 to 30 mg /kg. were fatal in 3 to 20 hours with incoordination, tremors, weakness and depression. The intravenous administration of 20 mg./kg. in one rabbit induced convulsions, running movements and terminal respiratory failure with death occurring at 8 minutes. In a second rabbit 10 mg./kg. caused extreme depression and several intermittent convulsions followed by death in 2 hours. Parasympathomimetic action was mild.

Bis(β-chloroethyl)methylamine: Within 5 to 15 minutes after intravenous injection in rabbts, 20 mg./kg. of HN2 caused marked incoordination accompanied by brief convulsive running movements, salivation, urination, defecation, lacrimation, bronchorrhea and miosis. The subcutaneous injection of 20 mg./kg. or more in mice was followed by depression, constant tremors and intermittent convulsive activity. In mice surviving longer, incoordinated movements, severe tremors, over-reaction to stimuli, coldness, diarrhea and retropulsive movements were observed.

Neurologic injury: In the absence of the usual signs of delayed systemic intoxication in rats, manifestations of neurologic injury have sometimes appeared on the 3rd or 4th day in rats after exposure to HN1 or HN2 vapor and after the intravenous administration of either agent. These manifestations consist of increased irritability, abnormalities of posture and movement, progressing in the severe cases to apparently severe involvement of the vestibular and cochlear mechanisms. Usually death rapidly followed the onset of these extreme effects, but among survivors with less severe injury hyperirritability, persisting for weeks, remained the only sign of injury. Pathologic examination showed extensive demyelination of the peripheral nerves in $\frac{1}{3}$ animals.[2] Although this lesion is

similar to that produced by vitamin B_1 deficiency, the rapid onset of the syndrome argues against this interpretation.

Tris(β-chloroethyl)amine: After intravenous injection of 10 to 30 mg./kg. of HN3, rabbits developed opisthotonic convulsions and died within 3 to 4 minutes. A dose of 6 mg./kg. induced front leg extension, backward retraction of the head, rearing and wild jumping or retropulsive movements terminating in loss of motor control. These paroxysms recurred at intervals, ending finally in gasping respiration and death from respiratory failure in a few hours.

Doses of 60 to 100 mg./kg. of HN3 given subcutaneously to mice produced incoordinated movements, hyperexcitability, and increased respiration culminating in convulsive seizures which ended with the hind legs extended and abducted, the neck flexed and terminally cessation of respiration.

Below doses which are immediately convulsive (6 mg./kg. intravenously in rabbits, 30 to 50 mg./kg. subcutaneously in mice) there developed progressively muscular weakness, diarrhea, coldness, hyperexcitability and overactivity, retropulsive movements, tremors and incoordination, and terminally prostration and failure of respiration. Convulsions occurred only in the terminal phase and were possibly related to medullary anoxia.

Derivatives of the nitrogen mustards. In dilute aqueous solutions the first reaction of the nitrogen mustards is the formation of a cyclical imonium ion. Subsequent reactions yield successively the β-chlorothyl-β-hydroxyethylamines, the β-hydroxyethyl-ethylenimonium ions, and finally the *bis(β-hydroxyethyl)-ethylenimonium* ion and *tris(β-hydroxyethyl)amine.* (For summary of these reactions see (4)). In addition to this type of reaction, in more concentrated solutions intermolecular reaction gives rise in the case of HN2 to N,N'-dimethyl-N,N'-*bis(β-chloroethyl)-piperazinium* dichloride, the cyclic dimer formed by reaction of two molecules of HN2 (5).

The pharmacologic properties of these derivatives must of course be considered in the over-all toxicity of the parent amines. Table 2 shows the toxicity of some of these derivatives. Because of the ease with which it is formed in the reaction of HN2 and sodium thiosulfate, the Bunte salt of HN2 is included in this table. The dimer of HN2 and the Bunte salt of HN2 are relatively non-toxic. Among the remaining compounds the order of decreasing toxicity is as follows: parent amine, β-chloroethyl-ethylenimonium salts, β-hydroxyethyl-ethylenimonium salts and β-chloroethyl-β-hydroxyethylamines. The final derivatives, the *bis(β-hydroxyethyl)amine* and *tris(β-hydroxyethyl)amine,* are non-toxic (6, 7).

Ethyl-β-chloroethyl-ethylenimonium picrylsulfonate: This compound does not possess marked parasympathomimetic action; large doses given intravenously to rabbits produce only mild transient salivation and no pupillary changes. When given intravenously or subcutaneously in mice or intravenously in rabbits it has a depressant action. Administered subcutaneously to mice and intravenously to rats at LD_{50} doses, it produces delayed deaths and its action is comparable to the parent amine with respect to weight loss, diarrhea and the degree of leucopenia. Rats do not show the neurologic syndrome seen after intravenous administration of the parent amine. In the rabbit, leucopenic action is no

greater than and possibly less than the parent amine, but weight loss and diarrhea are observed.

Ethyl-β-chloroethyl-β-hydroxyethylamine: This derivative seems to produce qualitatively the same effects as the parent amine. Large doses given intravenously, subcutaneously or intraperitoneally in mice produce symptoms ranging from tremors to convulsions accompanied by hyperirritability and incoordinated hyperactivity. At higher doses intravenously, gasping and momentary cessation of respiration occur. Lower intravenous doses (8 to 20 mg./kg.) cause delayed deaths similar to those seen in rats given HN1 intravenously. Lower subcutaneous (12 to 20 mg./kg.) and intraperitoneal doses (40 to 50 mg./kg.) produce deaths of a mixed type, 50 per cent of the animals dying within 2 hours and the remainder in 2 to 5 days, after the appearance of weakness, diarrhea and weight loss. Lower doses given intraperitoneally cause only delayed deaths.

In the rabbit higher doses cause depression, progressing until death between 12 and 16 hours in one instance (10 mg./kg.), but in the second instance (20 mg./kg.) depression was punctuated by a period of hyperexcitability, possibly a release phenomenon. Rabbits survived 5 mg./kg. and developed a mild leucopenia.

Ethyl-β-hydroxyethyl-ethylenimonium chloride: This compound administered to mice intravenously, subcutaneously or intraperitoneally at LD_{50} and supra-LD_{50} doses caused acute deaths, the animals progressing from depression to weakness and terminal respiratory convulsions. Administered intraperitoneally a significant number of delayed deaths occur among mice surviving the acute toxic effect. Depression may last for 24 hours. This derivative does not produce leucopenia and it possesses no parasympathomimetic action. Survivors show no weight loss.

Methyl-β-chloroethyl-ethylenimonium picrylsulfonate: Mice given large doses of this derivative intravenously or subcutaneously show parasympathetic effects, depression, weakness and death in from one minute to 12 hours depending upon the dose and route. Doses of 2.0 mg./kg. intravenously and 2.4 mg./kg. subcutaneously caused delayed deaths. Leucopenia occurs consistently in animals given lethal doses of this derivative.

Methyl-β-chloroethyl-β-hydroxyethylamine: The hydrochloride of this compound administered parenterally to mice gives rise after a latent period to depression, followed in 1 or 2 hours by terminal respiratory convulsions and death. Survivors of the immediate effects show no evidence of systemic intoxication and no delayed deaths were observed. In rabbits large doses given intravenously produce depression, severe muscular paralysis and early deaths. At lower doses paralysis is reversible. This derivative does not cause leucopenia. The toxic action appears to be predominantly on the central nervous system.

Prophylaxis in rabbits with nembutal (13 mg./kg., intravenously), urethane (1000 mg./kg. intraperitoneally), hexamethylenetetramine (1000 mg./kg. intravenously), and sodium thiosulfate (500 mg./kg. intravenously) gave definite evidence of protection against 2.5, 2, 2 and 2 LD_{50} doses, respectively, of methyl-β-chloroethyl-β-hydroxyethylamine administered intravenously. The action

of nembutal seemed qualitatively distinct from the other agents, in that it was effective in low doses, 7.5 mg./kg. affording some degree of protection against an approximate 2.5 LD_{50} dose of the amine. No antidotal value was evident when the nembutal was given therapeutically. In unanesthetized rabbits occlusion of the carotid arteries during and for 15 minutes after the intravenous injection of supra-LD_{50} doses of the amine afforded some protection, but this procedure is less effective than nembutal.

Methyl-β-acetoxyethyl-β-chloroethylamine: This compound is relatively non-toxic and the exact LD_{50} was not determined. The value is estimated to be in the range of 36 mg./kg. After injection at this dose mice were depressed and frequently showed mild recurrent convulsions. Fatalities were immediate (2 to 3 hours) and there was no evidence of delayed action. Judging from the immediate type of death methyl-β-acetoxyethyl-β-chloroethylamine probably exerts its action through the formation of methyl-β-chloroethyl-β-hydroxyethylamine.

Methyl-β-hydroxyethyl-ethylenimonium picrylsulfonate: This derivative possesses essentially the same pharmacologic properties as methyl-β-chloroethyl-β-hydroxyethylamine.

N,N'-dimethyl-N,N'-bis(β-chloroethyl)-piperazinium dichloride: HN2 dimer given to mice subcutaneously in doses of 2,500 to 5,000 mg./kg. induced slight convulsive twitching, paralysis and death in 12 to 13 minutes. Mice receiving 600 to 1000 mg./kg. lost weight and developed progressive prostration with extreme weakness, paralysis and death in coma at 57 to 160 hours. Mice receiving 400 mg./kg. showed temporary weight loss followed by recovery. Doses of 50 to 200 mg./kg. were without effect upon weight.

Disodium bis(β-thiosulfatoethyl)methylamine: Large doses of this derivative given to mice subcutaneously (1,000 mg./kg.) or intravenously (500 or 1,000 mg./kg.) and to rabbits intravenously (100 or 200 mg./kg.) caused convulsions of varying character depending upon the species and the route. The attacks were usually of a spastic nature, but in mice intravenously severe clonic convulsions were observed. Lower doses produced tremors, incoordination, and hyperirritability followed by weakness and depression followed by recovery. Intravenously administered in rabbits 50 mg./kg. caused no weight loss and no disturbance in daily total and differential white cell counts.

Compounds related to the nitrogen mustards. The compounds in table 3 are chiefly of interest in relation to the capacity of the nitrogen mustards to produce leucopenia and consequently most of them have been examined only for toxicity and leucopenic action. Two compounds, vinyl-β-(*bis*(β-chloroethyl)amino)-ethyl sulfone and N,N,N',N'-*tetrakis*(β-chloroethyl)ethylenediamine, were studied in more detail. These compounds are arranged in table 3 according to their chemical constitution. Several generalizations can be made from the data: (1) Stable quaternary salts (IIC1 and IIC2) do not induce leucopenia in spite of the presence of two β-chloroethyl groups in the molecule. (2) Compounds which can cyclize induce leucopenia if they contain two or more β-chloroethyl groups (e.g. IIA, IIB1, IIB2, IIB3). This group (2) contains a

secondary amine *bis(β-chloroethyl)amine* which on cyclizing would yield an imine, *β-chloroethyl-ethylenimine*, and judging by analogy with other compounds of this type, the cyclical imine would be highly reactive (8). (3) Compounds which can cyclize do not induce leucopenia if they contain only one β-chloroethyl group (e.g. IA1, IA2, IB1). The hypothesis that leucotoxic action is related to the ability to cyclize has been extended elsewhere (9) in a quantitative study correlating reactivity with toxicity.

Vinyl β-(bis(β-chloroethyl)amino)ethyl sulfone: The action of this compound in mice resembles that of divinyl sulfone and will be discussed elsewhere. However, in the rabbit this compound has a convulsant action. Of 14 rabbits injected intravenously with a dose of 2.67 mg./kg. or greater, 13 had convulsions. Only $\frac{1}{12}$ had convulsions at lower doses. The time of onset varied from 4 to 5 minutes at a dose of 9 mg./kg. to 22 minutes at a dose of 1.78 mg./kg. Death usually occurred in 15 to 155 minutes but 2 animals that were alive at 2 hours were found dead at 18 hours. Following injection and before the appearance of convulsions at the higher doses, the animals showed salivation, increased excitability, sometimes defecation and urination and always an increased respiration rate. Muscle tone progressively decreased and as the convulsive stage approached, the ears sagged to the side. Convulsions were severe with rapid running movements propelling the animal violently about the room. At termination of the clonic seizure, a short period of opisthotonus occurred and after the higher doses death terminated this stage. Among animals surviving the opisthotonic stage relaxation followed and in a few minutes consciousness returned. The conscious animal rights itself with difficulty, makes few volitional movements and appears extremely depressed. At a lower convulsive dose, convulsions may recur until death supervenes.

Convulsions have never been observed in mice injected intravenously or subcutaneously. In the rabbit convulsive action does not depend on the route of administration, since 2 rabbits injected subcutaneously with 3.8 mg./kg. had convulsions fully as severe as those seen in intravenously injected rabbits.

N,N,N′,N′-tetrakis(β-chloroethyl)ethylenediamine dihydrochloride: Two rabbits receiving 10 mg./kg. of this compound developed convulsions during intravenous injection and were dead within one minute. Three rabbits given 5 mg./kg. showed similar immediate reactions; 2 died within 20 minutes while the third survived for 5 days. Within 3 to 4 minutes after the injection these animals began to have clonic and tonic convulsions which recurred every few minutes. Five minutes after injection the animals showed jerking movements of the ears, grinding of teeth, rapid, noisy respiration, slight salivation, weakness of the forelegs, but no constriction of the pupils. These symptoms persisted and were followed by weakness of the hindlegs and inability to remain upright. The animal surviving for 5 days continued to have convulsions for at least 2 hours after the injection. On the third day there was a marked leucopenia, the reduction in total granulocytes being greater than the reduction in lymphocytes. Diarrhea developed prior to death but weight loss was slight (2 per cent).

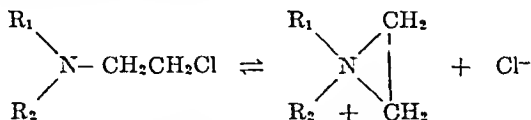Four rabbits receiving 3.3 mg./kg. intravenously had immediate reactions

similar to those described above. Intermittent convulsions persisted for at least 2 hours. Three of the 4 died at intervals from 5 to 18 hours. The remaining animal developed diarrhea on the 2nd day and this persisted until the 9th day. Maximal weight loss was recorded on the 9th day and recovery followed.

Of 5 rabbits given 2.2 mg./kg. intravenously, two had immediate convulsions and died within 7 minutes. A third had a few mild convulsions beginning 50 minutes after injection but survived. The remaining animals survived without signs of intoxication.

Rats receiving large doses (6.0 to 11.3 mg./kg.) of this compound intravenously had convulsions and died 15 to 60 minutes later. Among 6 rats receiving 4 mg./kg. there were no convulsions. Only one animal survived, the remainder dying after 1 hour to 4 days. Of 6 rats given 3.3 mg./kg., one died 5 hours and another 48 hours after injection. Three other animals died by the 6th day of delayed effects. Apart from weight loss rats receiving lower doses showed no signs of intoxication. No deaths occurred in the first 24 hours after subcutaneous administration.

DISCUSSION. The ability to produce the systemic injury and delayed death characteristic of the nitrogen mustards is restricted to compounds containing two β-chloroethyl groups attached to a nitrogen (or sulfur atom) and capable of forming cyclical onium ions. Cytotoxic action is probably the result of the alkylating property of the several onium ions which may result from the hydrolysis of a single compound. Neurologic action may result from the localization of alkylation within nerve cells or may result from the action of quaternary salts at synapses. The chemical kinetics of such compounds indicate what may be expected when these agents are introduced into the body.

Slightly acid solutions of the hydrochlorides of the nitrogen mustards are stable. However, under physiological conditions (pH 7.5, 37°C.) these compounds rapidly cyclize and give rise to the ethylenimonium ion, according to the reaction proceeding to the right:

$$\begin{array}{c} R_1 \\ \diagdown \\ N-CH_2CH_2Cl \\ \diagup \\ R_2 \end{array} \rightleftharpoons \begin{array}{c} R_1 CH_2 \\ \diagdown \diagup | \\ N | \\ \diagup \diagdown | \\ R_2 + CH_2 \end{array} + Cl^-$$

This reaction is monomolecular and depends only upon the concentration of the amine. Thus, when the amine enters body fluids by any route, it may be expected that the cyclical imonium ion will be formed. This ion is very reactive and may enter into reactions which for the purpose of the present discussion may be divided into 3 types: (A) Being a powerful alkylating agent, it reacts with free amino, carboxyl and sulfhydryl and other susceptible groups present in biological systems. This type of reaction will of course occur in both the intracellular and extracellular fluid; (B) The imonium ion reacts with water, with the production of the chlorhydrin (the alkyl-β-chloroethyl-β-hydroxyethylamines); (C) The imonium ion reacts with chloride, in accordance to the reaction

to the left in the above equation. This reaction is biomolecular and its rate depends upon the concentration of both the imonium ion and chloride, the rate of reaction C being increased in the presence of chloride, as for example in extracellular fluid. Even though chloride shifts the equilibrium to the left, the imonium ion is nevertheless formed at a rate solely dependent upon the concentration of the amine. Thus, chloride competes with water and with organic radicles subject to alkylation for such imonium ion as is present. But because of the chloride present in extracellular fluid there will be considerably more of the amine present than there will be in aqueous solutions buffered to the same pH.

This fact is important if it is supposed that it is the free amine and not the imonium ion that enters cells, since cell membranes are relatively impermeable to ionic substances. The toxicity and action of the imonium salts of HN1 and HN2 support this view. For example, the imonium salt of HN2, methyl-β-chloroethyl-ethylenimonium picrylsulfonate is as toxic as the parent amine and produces leucopenia. If this salt cannot enter cells, its cellular action must be accomplished by some other derivative formed in the extracellular fluid. Exclusive of those reaction products formed by alkylation of organic substances[3], only 2 derivatives are possible. These are the parent amine formed by reaction C, and methyl-β-chloroethyl-β-hydroxyethylamine formed by reaction of the imonium ion with water. The action of the imonium ion cannot be propagated through the latter of these derivatives since leucopenia is not produced by the chlorohydrin. Therefore, it seems apparent that leucopenic action of the imonium is attributable to the re-formation of the parent amine by reaction with chloride.

These considerations suggest that high reactivity of the imonium ion (particularly with water) reduces cytotoxic effects for the reason that such imonium ions are rapidly destroyed in extracellular fluid. Therefore, considering only their behavior in extracellular fluid, the most potent cytotoxic agents should be those whose imonium ions have a low reactivity with water.

Having entered the cell, the amine again cyclizes. Within the cell, however, there is little, if any, chloride ion to compete along with water for the imonium ion against cellular constitutents containing labile groups which may be alkylated. Thus, conditions here are more favorable for alkylating reactions, and it seems apparent that high reactivity towards groups susceptable to alkylation enhances cytotoxic action. High reactivity towards water would, however, destroy the alkylating agent.

Great import attaches to the question of reaction of imonium ions with cell membranes from the exterior. The reaction of the imonium ion with free groups lying on the exterior surface of cell membranes certainly merits consideration. Such a reaction could alter membrane permeability and permit the cytotoxic agents easier access to the interior of the cell as well as permit egress of cellular constituents. This view is supported by some evidence. Thrombophlebitis is more severe at the site of injection of solutions containing predominantly

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[3] The possible participation of a non-ionic derivative formed by the alkylating process and capable of crossing cell membranes cannot be excluded with certainty. However, none of the derivatives so far studied have lent any support to this hypothesis

methyl-β-chloroethyl-ethylene-imonium chloride than when the parent amine was given similarly. Marked unilateral edema was observed in tissues receiving blood peripheral to the site of intracarotid injection of a similar solution of the imonium ion. In dogs given 20 mg./kg. of *tris*(β-chloroethyl)amine on the skin increased catabolism as judged by nitrogen excretion accounts for only 50 per cent of the increased potassium excretion. On the other hand experiments on dogs intoxicated in this laboratory with HN3 have indicated that increased capillary permeability, if present, is so limited as to fall within the error of the experimental method. Thus, the contribution of such a reaction in producing systemic injury remains obscure. (Model experiments with amino acids, proteins and other cellular constituents warrant the hypothesis that some alkylating reaction is responsible for the initial injury or ultimate death of the cell.)

These experiments have afforded no information on the nature of the injury process or on why some cells (lymphoid tissue, bone marrow, intestinal epithelium, etc.) are particularly sensitive to the action of the β-chloroethyl derivatives.

SUMMARY

The toxicity and pharmacological action of the nitrogen mustards and certain related compounds have been studied in various species. From study of the related compounds it is apparent that the toxicity and ability to produce delayed deaths and leucopenia requires the presence of 2 β-chloroethyl groups and the formation of a cyclical imonium ion. Among the hydrolytic derivatives of the nitrogen mustards pharmacological action of high doses which produce acute deaths does not contribute significantly to the systemic injury which produces delayed deaths at lower doses.

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ANTAGONISM OF CURARE ACTION BY NEOSTIGMINE, PHYSOSTIGMINE, EPHEDRINE AND DI-ISOPROPYL FLUOROPHOSPHATE (DFP)

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In accordance with the theory of chemical mediation of impulse transmission at the neuromuscular junction, neostigmine has come to be regarded as an effective antidote to curarization. It has been considered that its activity in that respect is largely or solely due to its ability to inhibit cholinesterase, in this manner permitting the concentration of acetylcholine to increase to a level sufficient to break through the curare block.

In several reports (1, 2, 3) the use of 'Intocostrin', a standardized preparation of curare, in anesthesia is described. Comment on the possible antidotal use of neostigmine is often made in these reports, although occasions for its use did not occur.

Many substances have been proposed as curare substitutes. In studying the action of some of these, one of us (4) gained the impression that neostigmine was without benefit as an antidote to the paralysis caused by quaternary quinine salts. This was in contrast to the experience of Bennett and Cash (5), who used quinine methochloride in shock therapy of mental disorders. These workers observed that neostigmine could be used to counteract the respiratory paralysis which occurred from overdosage with this quinine derivative. Because of this difference in experience it was felt that the efficacy of inhibitors of cholinesterase as antagonists to the curarizing action of various curarizing agents merited more extensive study.

In this investigation studies have been made of the antagonism between neostigmine and such curariform drugs as 'Intocostrin', d-tubocurarine, dihydrobetaerythroidine, quinine methochloride, quinine ethochloride, tetramethylammonium chloride, and tetraethylammonium chloride.[2] The quaternary ammonium salts were included for comparison as the simplest type of onium salts reported to have curarizing activity (6). Physostigmine was also tested for its antagonistic action when given in conjunction with quinine ethochloride or d-tubocurarine. Since di-isopropyl fluorophosphate[3], an inhibitor of cholinesterase, has been shown to exert some beneficial effect on the muscular weakness

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of myasthenia gravis (7), its influence on the action of curarizing drugs was studied. The possibility of synergistic action between ephedrine or atropine and the drugs inhibiting cholinesterase was also investigated.

METHOD. The rabbit head-drop assay was carried out in a manner similar to that used by Holaday (8) in the bioassay of 'Intocostrin', as elaborated in the Squibb Laboratories, with the exceptions that the animals were injected while sitting unrestrained in a U shaped enclosure and a "priming" dose of the curarizing agent, representing from one-fourth to one-third of the estimated head-drop dose, was given initially. After an interval of one minute, small aliquots of 0.05 cc. were administered intravenously every fifteen seconds. An exception was made in the assay of quinine methochloride which, because of its insolubility, was prepared in lower concentration and injected in 0.1 cc. increments. Rapid intravenous injection was employed for the antagonists studied. They were given alone or in combinations one minute before beginning the administration of the curarizing agent.

The cross-over technique was used in the studies of the effective dosage ranges of neostigmine and physostigmine with and without atropine, otherwise control "titrations" were performed on a single day and test "titrations" repeated on the same animals from one to three days later.

Strengths of solutions of curarizing agents were chosen so that the total time required for injection of each drug would be similar for all the paralysants studied. These drugs, dissolved in water, had the following concentrations: 'Intocostrin', 4.0 units per cc.; d-tuhocurarine chloride, 4.0 units per cc. (0.65 mgm. per cc.); dihydrohetaerythroidine hydrobromide, 5.0 mgm. per cc.; quinine methochloride, 15 mgm. per cc.; quinine ethochloride, 20 mgm. per cc.; tetramethylammonium chloride, 10 mgm. per cc.; tetraethylammonium chloride, 200 mgm. per cc. Neostigmine methylsulfate and physostigmine salicylate were used in the form of solutions containing 0.5 mgm. per cc.; ephedrine in 10 mgm. per cc. Because of its instability (9), fresh solutions of di-isopropylfluorophospate, 1.0 mgm. per cc., were prepared immediately before injection. Atropine sulfate was administered subcutaneously in 5 and 10 mgm. per kgm. doses one half to one hour before the titrations of the curarizing agent. The animals were allowed to rest for one week for the effects of the atropine to be dissipated before studies were continued. These large doses were chosen for atropine because of the known resistance of the species to the drug.

In each test from 9 to 24 rabbits were used. The results of each assay were averaged and were subjected to statistical analysis, using methods for correlated data in comparing the head-drop dose following antidotal agents with the control head-drop dose in the same animal. A critical ratio of 2.0, indicating that the difference between the means of the two groups of data under consideration was at least twice the standard error of that difference, was considered to denote significance.

RESULTS. *Action of curarizing agents.* Repeated determinations of the head-drop doses of the same curarizing agent on consecutive days revealed some variation in the response of individual animals (table 1). These variations failed to produce significant difference between the means of any two control studies of a single drug, when the data were derived from at least nine animals. This is confirmed in table 2, which summarizes the preliminary assays with the various curarizing agents.

Each milligram of d-tubocurarine is stated by the manufacturer to contain 6.17 'Intocostrin' head-drop units (or 1 unit = 0.16 mgm.); the average head-drop dose of d-tubocurarine found in several hundred determinations in our laboratory was approximately 0.2 mgm. per kgm. This difference between the assay of the manufacturer and the present assay appeared to be due to the fact

that, in this study, animals were injected while in a "sitting" position with only gentle manual restraint, rather than tied to a board in a prone position. We have found that rabbits restrained in a prone position usually exhibit head-drop doses that are lower by about 20 per cent, more nearly equivalent to the manufacturer's assay.

Some of the animals curarized with dihydrobetaerythroidine appeared to have marked bronchospasm with accompanying cyanosis, preceding the development of head-drop. In three of ten rabbits, cyanosis was followed by convulsions each time they were injected with the drug. Recovery from head-drop occurred more rapidly after the quinine salts than following the administration of 'Intocostrin' or the curare alkaloids.

Preceding the curarizing action, tetramethylammonium chloride exerted

TABLE 1

Rabbit head-drop assay

'Intocostrin' and Neostigmine-'Intocostrin' (Hd. Dr. Dose = units/kg.)

RABBIT NO	CONTROL #1	CONTROL #2	WITH NEOSTIGMINE 0 0 MGM /KGM.
1	1.0	0.9	2.0
2	1.3	1.5	2.7
3	1.6	1.4	2.7
4	1.8	1.8	3.4
5	1.7	1.3	3.4
6	1.4	1.0	2.2
7	1.3	1.5	2.5
8	1.1	1.1	3.5
9	0.9	1.2	2.7
10	1.8	2.0	4.6
Mean hd. dr. dose	1.39	1.37	2.97
Standard deviation . . .	0.32	0.34	0.77
Standard error	0.10	0.11	0.24
Critical ratio		0.22	7.09

marked muscarinic effect, as noted by Burn and Dale (10). This was evidenced by copious salivation, myosis, lacrymation, micturition, and bradycardia. Inhibition of respiration, mentioned by Marshall (11) and by Loevenhart (12), followed each additional injection of this drug and lasted for about five seconds. Struggling, crying, and apparent attempts by each rabbit to brush the eyes with the forepaws also followed the injection. These various side-effects tended to obscure the endpoint of the "titration" with this compound. Head-drop doses ranged from 3.06 to 4.66 mgm. per kgm.

Tetraethylammonium chloride is known to be a relatively weak curarizing agent (13), and the dose which produced head-drop was found generally to exceed the fatal dose and to be about twenty times that for the tetramethyl analogue. Death, preceded by cyanosis and convulsions, followed within ten to twenty minutes after the appearance of head-drop. A few animals given doses

smaller than those necessary to produce immediate head-drop showed head-drop as a delayed reaction fifteen to twenty minutes after the injection was stopped.

Antagonism of curare action by neostigmine. The prior injection of neostigmine (0.05 mgm. per kgm.) raised the mean head-drop dose of 'Intocostrin'

TABLE 2

Antagonism of curare and curare-like agents by neostigmine 0.05 mgm./kgm.

CURARIZING AGENT	STATISTICAL DETERMINATION	MEAN 1ST CONTROL	MEAN 2ND CONTROL	CRITICAL RATIO TWO CONTROLS	AVERAGE CONTROLS	MEAN WITH NEOSTIGMINE 0.05 MOM./KGM.	CRITICAL RATIO AV. CONTROLS VS. NEOSTIGMINE
'Intocostrin'	*Hd. dr. dose**	1.39	1.37	0.22	1.38	2.97	7.09
	No. animals	10	10		10	10	
	Std. deviation	0.32	0.34			0.77	
	Std. error	0.1	0.11			0.24	
d-tubocurarine	*Hd. dr. dose*	0.175	0.192	0.72	0.201	0.495	10.1
	No. animals	9	9		16	16	
	Std. deviation	0.059	0.067			0.117	
	Std. error	0.017	0.022			0.029	
Dihydrobeta-erythroidine	*Hd. dr. dose*	1.75	2.00	1.26	1.88	5.99	7.6
	No. animals	10	10		10	10	
	Std. deviation	0.84	0.90			1.98	
	Std. error	0.26	0.28			0.62	
Quinine etho-chloride	*Hd. dr. dose*	6.32	6.02	0.83	6.17	6.56	0.98
	No. animals	20	20		20	20	
	Std. deviation	1.51	1.65			2.23	
	Std. error	0.33	0.36			0.50	
Quinine metho-chloride	*Hd. dr. dose*	6.3	6.84	1.23	6.57	5.98	1.77
	No. animals	18	18		18	18	
	Std. deviation	1.55	1.64			1.80	
	Std. error	0.36	0.38			0.42	

* All doses are in mgm. per kgm. except for 'Intocostrin' (units per kgm.).

to a value approximately two times, of d-tubocurarine to two and one-half times, and of dihydrobetaerythroidine to three times that of the averages of the respective control assays (table 2). However, neostigmine, in this dose, had little effect on the size of the average curarizing dose of quinine ethochloride and ap-

peared to decrease that of quinine methochloride; neither change could be considered significant as measured by the statistical criteria established.

This study was extended to include doses of neostigmine above and below 0 05 mgm. per kgm. with subsequent titrations with d-tubocurarine and quinine ethochloride (fig. 1). Marked increases in curarizing doses of d-tubocurarine occurred at 0.0125, 0.025, 0.05, and 0 075 mgm. per kgm. of neostigmine. Signs of toxicity appeared in some of the animals on the highest dose. Antagonistic action occurred with the quinine salt only at low dosages (0.0125 and 0 025 mgm per kgm.) of neostigmine.

Animals premedicated with neostigmine became extremely responsive to very small doses of tetramethylammonium chloride   Convulsions were frequent and doses that previously caused head-drop, or which were without effect, were lethal after neostigmine.   Because the curarizing effect of tetraethylammonium chlo-

TABLE 3

Antagonism of d-tubocurarine curarization by neostigmine and ephedrine

TEST	DOSE	NUMBER ANIMALS	MEAN HD DR DOSE	STD DEVIATION	STD ERROR	CR	CRITICAL RATIO TEST VS
	mgm /kgm		*mgm /kgm*	*mgm /kgm*	*mgm /kgm*		*mgm /kgm*
Control d-tubocurarine		19	0 227	0 04	0 009		
Neostigmine.	0 025	10	0 391	0 080	0 025	6 8	Control
Ephedrine	2 5	19	0 258	0 087	0 02	1 7	Control
Neostigmine plus ephedrine	0.025 1 0	10	0 464	0 144	0 04	0 45	Neostigmine 0 025
Neostigmine	0 05	14	0 501	0 125	0 033	8 5	Control
Neostigmine plus ephedrine	0 05 2 5	14	0 537	0 096	0 025	1.13	Neostigmine 0 05

ride was so difficult to demonstrate by the head-drop technique, antagonism of this action of the drug by neostigmine was not studied.

Antagonism of curare action by Physostigmine. Similar studies were made of the relationships between physostigmine in doses ranging from 0 05 to 0 25 mgm. per kgm. and d-tubocurarine or quinine ethochloride (fig. 2). The results were very similar to those with neostigmine. The percentile increase in head-drop dose following physostigmine was uniformly greater for d-tubocurarine than for quinine ethochloride   Similarly, also, the greatest increase occurred at a higher dose for d-tubocurarine than for the quinine derivative. This observation led to the question as to whether the muscarinic actions of neostigmine and physostigmine might, in some way, alter the action especially of the quinine congener.

Role of atropine. Premedication with atropine (5.0 or 10.0 mgm. per kgm. subcutaneously) one-half to one hour before "titration" of the curare agent lowered the control head-drop doses (atropine plus curarizing agent) to 60 to 70 per cent

of the control value for quinine ethochloride alone and to 90 to 95 per cent for d-tubocurarine alone (figs. 1 and 2). The contour of the graphs showing per cent increase of head-drop doses due to additional injection of neostigmine (atropine plus neostigmine plus curarizing agent), or of physostigmine, was similar to the contour of their respective curves showing activity with antagonist, but in the absence of atropine. The action of atropine in these doses was apparently to increase the effectiveness of the curare agents. This synergism was evident whether antagonist was given concurrently or not. The sole exception was in the case of the sequence, atropine plus physostigmine (0.175 mgm. per kgm.) plus d-tubocurarine, in which the effects were essentially the same as without atropine premedication.

Role of ephedrine. A detailed study of the antagonism of curare by neostigmine and ephedrine was undertaken, employing d-tubocurarine as the cura-
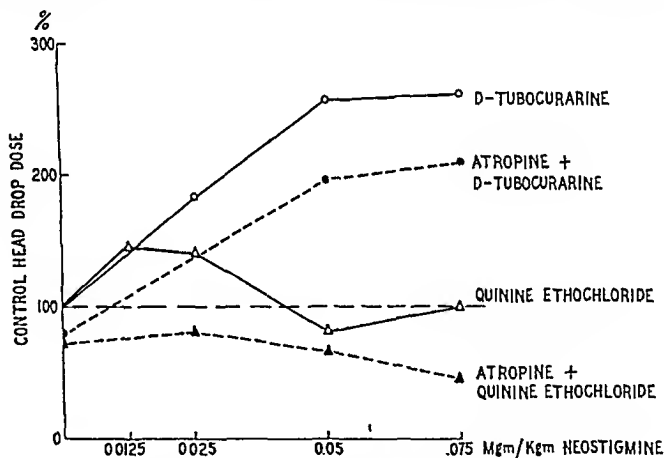
FIG. 1. NEOSTIGMINE AND CURARIZING ACTION

rizing agent. The results of this part of the investigation are outlined in table 3. Ephedrine sulfate, 2.5 mgm. per kgm., raised the endpoint of the assay insignificantly. The slight increase observed could be attributed mainly to the responses of two of the nineteen rabbits. Given concurrently with neostigmine, ephedrine had no significant effect on the head-drop dose of the curarizing drug.

Curare-like action of neostigmine. Koppanyi (14) and others have suggested that in large doses neostigmine has a curare-like action. When rabbits were "titrated" to head-drop with neostigmine, in the same manner as with the curarizing drugs, it was found that head-drop occurred at a mean dose of 0.122 mgm. per kgm. This dose is approximately 60 per cent greater than the highest dose used to antagonize curare action. Fasciculation and profuse salivation occurred during the titration before the endpoint was reached and six of the seventeen animals died in convulsions. The head-drop produced by neo-

stigmine is so confused by the muscarinic effects of the drug that the assay cannot be considered as conclusive evidence of curarizing action.

Antagonism of curare action by DFP. Di-isopropyl fluorophosphate (DFP) compared very closely in its action on neuromuscular transmission with the characteristic effects of neostigmine and physostigmine (fig. 3). Here, also, the peak effect occurred at a lower dose of DFP in combination with quinine ethochloride than with d-tubocurarine. The maximal antagonistic effect was similar
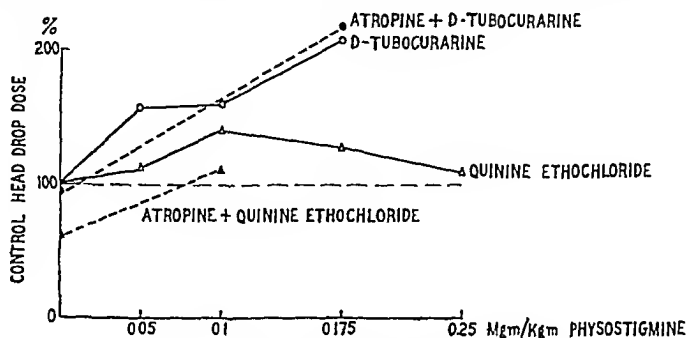
FIG. 2. PHYSOSTIGMINE AND CURARIZING ACTION
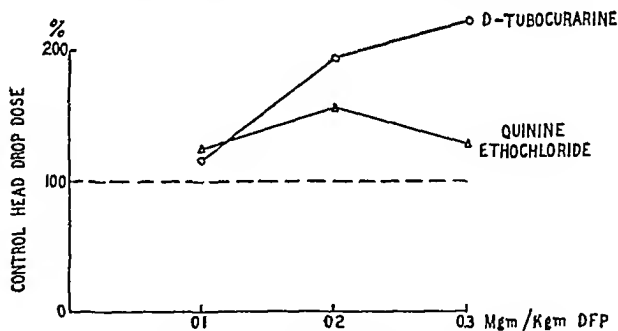
FIG. 3. DFP AND CURARIZING ACTION

in degree to that with physostigmine. DFP was also successful in raising the dose of quinine methochloride to 113 per cent of the control (critical ratio = 2 5).

Some information characterizing the antagonism between DFP and d-tubocurarine has been published in a preliminary report (15) and will be expanded more fully elsewhere. In this study there was no statistical difference in the degree of increase of head-drop dose whether the time interval between intravenous injection of DFP (0 2 mgm. per kgm.) and subsequent "titration" with curare be 1 minute, 5 minutes, or 1 hour. Assays in these same animals revealed a slow return to normal head-drop dose over a period of from 10 to 14 days.

DISCUSSION. We have been able to confirm the antagonistic action of neo-

stigmine for the partially purified curare preparation, 'Intocostrin', for its principal alkaloid, d-tubocurarine; and for another naturally occurring alkaloid, dihydrobetaerythroidine. We have also shown the comparative efficacy of the other cholinesterase inhibitors, physostigmine and diisopropyl fluorophosphate in inhibiting the effects of d-tubocurarine. It is worthy of note that the most effective doses of each of the antagonists raised the rabbit head-drop doses from 100 to 160 per cent above control values. The similarity of effect on curare action of three compounds of such similar physiologic activity, but of such dissimilar chemical nature would suggest that curare action is, indeed, related to acetylcholine activity at the neuromuscular junction and therefore is indirectly affected by the cholinesterase inhibitors.

There appears to be a difference between the antidotal properties of the inhibitors for the true curare alkaloids and for such curare substitutes as the quaternary quinine derivatives. The percentile increase in head-drop dose provided by these antagonists is not as large for quinine ethochloride as for d-tubocurarine. Moreover, the dose of each inhibitor providing maximal antagonistic effect for quinine ethochloride falls below the similar dose maximally antidoting d-tubocurarine, and is well below the dose of neostigmine, physostigmine, or DFP at which toxic side effects usually become apparent.

These findings suggest that quinine ethochloride may have some mechanism of muscular paralysis other than solely by blocking acetylcholine. Such an action might not be antagonized by cholinesterase inhibitors. The parent alkaloid, quinine, is known to produce a change in the muscle action potential which is presumed to be due to an effect directly on the muscle cell (16). The fundamental character of the tertiary alkaloid may not be entirely lost, as in the instance of the quaternary atropine salt described by Crum-Brown and Fraser (6). We were unable to show by the rabbit assay method that quinine hydrochloride was synergistic with the curarizing action of quinine ethochloride. Quinine hydrochloride was given as a "priming" dose equivalent to half the estimated curarizing dose of the quaternary derivative, and also was employed in attempts to complete curarization after priming with quinine ethochloride, but without effect. An alternative explanation might be that quinine derivatives synergize the muscarinic actions of the inhibitors and thus produce toxic states at lower doses. This situation would seem to be improbable in the light of our findings with atropine. A third possible explanation might be that a cardiac effect of the quinine derivative occurred similar to quinidine action. The heart, however, did not seem to be especially slowed in these experiments except when respiratory paralysis and attendant anoxia occurred.

A fourth possibility is suggested by Nachmansohn's observation (17) that quinine is itself a weak inhibitor of cholinesterase. The chance occurs, therefore, that concentrations of quinine ethochloride sufficient to produce head-drop might have already inhibited a portion of the cholinesterase. Under these circumstances the control head-drop value would be inordinately high and addition of other inhibitors of cholinesterase would have less apparent activity when calculated on a percentage basis.

The calculation of dosages on a molar basis offers some interesting points for speculation. The dosages studied (Figs. 1, 2, 3) ranged in multiples of 10^{-7} mols per kgm. from 0.36 to 2.2 for neostigmine, from 1.2 to 6 0 for physostigmine, and from 5.9 to 17.8 for DFP. Maximal antagonism of d-tubocurarine occurred at 2.2, 4.2, and 17.8 $\times$ 10^{-7} mols per kgm. respectively. Thus they were effective in the approximate ratio of neostigmine, 2; physostigmine, 4; and DFP, 16. Correspondingly maximal antagonism of quinine ethochloride occurred at doses which were in the approximate ratio of 1:4:15. When a 0.025 mgm. per kgm. (0.73 $\times$ 10^{-7} mols per kgm) dose of neostigmine was given as premedication, an additional 4 molecules of d-tubocurarine were necessary for every molecule of antagonist; at 0.05 mgm. per kgm. (1.46 $\times$ 10^{-7} mols per kgm.) the relationship was 3 additional molecules of this curare alkaloid to one of neostigmine and at 0.075 mgm. per kgm. (2.2 $\times$ 10^{-7} mols per kgm.) the ratio was 2 molecules to 1 of neostigmine. For the three doses of physostigmine the ratios were, respectively, 1.5, 0.8 and 0.8 extra mols of d-tubocurarine for each mol of physostigmine. These ratios would suggest that the antagonism is less efficient at the larger doses. This change might be expected from the fact that neostigmine, in sufficiently large dosage, appears to act in similar manner with curare, as we have confirmed. The curarizing dose of quinine ethochloride is fifty times greater than that of d-tubocurarine on a molar basis.

The findings reported here do not clearly demonstrate efficacy of ephedrine as a sole antidotal agent to curarizing action (table 3). When epehedrine was given in conjunction with neostigmine, the beneficial action of ephedrine was not demonstrated to any convincing degree. It is possible that had the experiments been carried out with the animals in extremis, at the lethal dose level, as were Koppanyi's (14), other factors in ephedrine's action might have been in evidence.

The authors wish to thank Dr. Huldah Bancroft for her kindly advice and helpful criticism of the statistical analyses of the results obtained in this study.

SUMMARY AND CONCLUSIONS

1. The rabbit head-drop method was used to assay the curarizing potencies of 'Intocostrin', d-tubocurarine, dihydrobetaerythroidine, quinine methochloride, quinine ethochloride, tetramethylammonium chloride and tetraethylammonium chloride.

2. Neostigmine raised the head-drop doses of 'Intocostrin', d-tubocurarine, and dihydrobetaerythroidine significantly. In low dosage neostigmine raised the head-drop doses, but at higher doses it failed to raise, or even slightly lowered, the curarizing doses of quinine etho- and methochloride

3. Physostigmine produced effects similar to neostigmine.

4. Subcutaneous preadministration of atropine in large doses tended to lower head-drop doses whether in control assays or in the presence of antagonists.

4. Ephedrine appeared to have little antagonistic action to curarization by d-tubocurarine, either when used as the sole antidotal agent or in combination with neostigmine.

6. Di-isopropyl fluorophosphate (DFP) was antagonistic to the curarizing action of d-tubocurarine, as well as to that of quinine metho- and etho-chloride.

7. The toxicity of tetramethylammonium chloride was increased by the previous adminstration of neostigmine. The curarizing properties of tetraethylammonium chloride were so weak that this compound could not be satisfactorily assayed by the head-drop technique.

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THE ESTIMATION OF 4'-CARBOXYPHENYLMETHANE SULFONANILIDE (CARONAMIDE) IN BIOLOGICAL FLUIDS

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4-Carboxyphenylmethanesulphonanilide (caronamide) inhibits reversibly the secretion of the penicillins by the kidney tubules (1, 2) and thus may be useful clinically for achieving higher and sustained plasma penicillin levels. Quantitative studies on the pharmacological effect and physiological disposition of caronamide required the development of a method for its estimation in biological fluids. A simple and accurate method is described here.

Caronamide is separated from the acidified biological material by extraction into chloroform. The drug is then returned to 0.1 N NaOH and the concentration of the compound is estimated spectrophotometrically at a wavelength of 280 μ. The procedure is specific to the extent that it is subject to negligible interference from normally occurring substances or metabolic products of caronamide.

A simplified procedure involves the deproteinization of the plasma with cadmium hydroxide and the measurement of the caronamide in the resulting filtrate. This procedure is inherently less specific than the extraction procedure since metabolic products of caronamide contribute to the measurement.

EXTRACTION PROCEDURE. *Reagents.* 1. Standard solution of caronamide, 100 mgm. per liter. 100 mgm. of the drug are dissolved in 1 liter of 0.1 N NaOH.

2. 1 N HCl.

3. Chloroform. A reagent grade of chloroform is purified by shaking successively with 1 N NaOH and 1 N HCl, followed by two washes with water.

4. 0.1 N NaOH.

Add 1 ml. of plasma or diluted urine and 1 ml. of 1 N HCl to 30 ml. of chloroform in a 60 ml. glass stoppered bottle and shake for 30 minutes on a shaking apparatus. Transfer the contents to a test tube and centrifuge at 3000 R.P.M. for 5 minutes. Remove the aqueous layer by aspiration. Transfer 20 ml. of the solvent phase to a 60 ml. glass stoppered bottle containing 5 ml. of 0.1 N NaOH. Shake for 3 minutes and then centrifuge at 3000 R.P.M. Transfer about 3 ml. of the aqueous phase to a quartz cuvette and read the optical density in a spectrophotometer (Beckman quartz photoelectric spectrophotometer) with the instrument set at the wavelength 280 mμ. A reagent blank, with water substituted for plasma, is run through the same procedure.

Working standards of the drug are prepared by dilution of the stock standard with 0.1 N NaOH. The samples give readings on the spectrophotometer which are directly proportional to their concentration. A "dummy" consisting of 0.1 N NaOH is used for the blank setting of the spectrophotometer. A concentration of caronamide of 8 gammas per ml. should read about 0.515 in terms of optical density.

RESULTS. Table I contains a summary of recoveries of known amounts of caronamide added to plasma and urine. The results indicate that the sensitivity and precision of the method are adequate for amounts of caronamide as low as 25 gammas.

Analyses run on plasma and urine samples of caronamide over a period of several days gave highly reproducible results. It may be concluded therefore that caronamide in plasma and urine is stable when stored in the refrigerator.

TABLE I

Recovery of caronamide added to plasma and urine

PLASMA			URINE		
Caronamide added	Caronamide found	Recovery	Caronamide added	Caronamide found	Recovery
gammas	*gammas*	*per cent*	*gammas*	*gammas*	*per cent*
25	24	96	25	25	100
	24	96		23	92
	23	92			
50	47	94	50	49	98
	48	96		50	100
	52	104		50	100
	50	100		50	100
	49	98		51	102
	48	96		50	100
				48	96
				49	98
				49	98
				51	102
				50	100
100	94	94	100	94	94
	91	91		97	97
	98	98			
	98	98			
	100	100			
	94	94			
	95	95			
	94	94			
	92	92			

SPECIFICITY. The degree of specificity is dependent on the extent to which normally occurring metabolites and metabolic derivatives of caronamide are excluded.

There is a blank in dog plasma which amounts to about 0.4 mgm. percent. The dog urine blank is negligible. The possible interference of metabolic products of caronamide in the dog was examined by a general technique previously described (3). This is based on a comparison of the solubility characteristics of the pure compound with those of the material extracted from urine and plasma obtained from a dog to whom the compound was administered. It is clear from the data shown in Table II that chloroform extracts of plasma and urine contain

negligible amounts of material which absorb light at 280 mμ and which differ in solubility characteristics from pure caronamide.

PRECIPITATION PROCEDURE. *Reogents.* 1. Cadmium sulfate solution. 20.8 grams of 3 CDSO$_4$.8H$_2$O and 101.3 ml. of 1 N H$_2$SO$_4$ are dissolved in water and the resulting solution is diluted to 1 liter.

2. Sodium hydroxide solution, 1.1 N.

Into a 25 ml. erlenmeyer flask containing 1 ml. of plasma or diluted urine, 5 ml. of CDSO$_4$ reagent, and 3 ml. of water, add dropwise with shaking, exactly 1 ml. of 1.1 N NaOH. After 30 minutes, transfer to a test tube and centrifuge at 3000 R.P.M. for 5 minutes. Transfer

TABLE II

Distribution of caronomide and opparent coronamide betwcen woter and chloroform at vorious pH volucs

The apparent caronamide was obtained by extraction with chloroform of the plasma and urine of a dog, 1 hour after receiving the compound. The compound was then returned to dilute alkali. Aliquots of this solution and a caronamide solution were adjusted to various pH values and shaken with equal volumes of chloroform. The fraction of the compounds extracted at various pH values is expressed as the ratio of the amount of compound in the organic phase to total compound.

pH	PLASMA		URINE	
	Aqueous control (a)	Apparent caronamide from plasma exrract (b)	Aqueous control (c)	Apparent caronamide from plasma extract (d)
3 0	91	91		
4 7	71	76	70	75
5 0	51	50	44	52
6.0	11	10	10	11
7.0			3	4

Columns (a) and (c) pure caronamide; column (b) apparent caronamide from the plasma of a dog, extracted into chloroform and returned to dilute alkali; column (d) apparent caronamide from the urine of a dog, extracted into chloroform and returned to dilute alkali. For columns (a) and (h) measurements were taken at temperatures which differed from those for columns (e) and (d) by several degrees which accounts for the difference in the two sets of measurements.

5 ml. of the clear supernatant fluid to a test tube containing 1 ml. of the 1.1 N NaOH and mix thoroughly. A slight precipitate often occurs at this point and may be separated by centrifugation. Transfer about 3 ml. of the clear supernatant fluid to a quartz cuvette and read the optical density in a spectrophotometer with the instrument set at the wavelength 280 mμ. A reagent blank with water substituted for plasma is run through the same procedure.

The readings are compared with those of standards as described above.

Recoveries of known amounts of caronamide from urine are quantitative. Plasma recoveries are between 85 and 90 per cent.

This method is not specific. There is a plasma blank in dog plasma of the order of 2 mgm. per cent. The interference of metabolic products of caronamide in dog plasma results in an error of about 25 per cent.

SUMMARY

Simple spectrophotometric methods are described for the estimation of caronamide (4'-carboxyphenylmethane sulfonanilide) in biological fluids.

Caronamide is isolated from biological material by an extraction with chloroform at an acid pH. The compound is then returned to alkali and the concentration measured spectrophotometrically at 280 mμ. This method is specific in the case of the dog in that metabolic derivatives of caronamide do not interfere.

Another procedure measures the caronamide spectrophotometrically in protein filtrates of plasma. This method is simpler but less specific than the extraction procedure in that metabolic derivatives of caronamide interfere in the measurement.

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THE RENAL EXCRETION OF 4'-CARBOXYPHENYLMETHANE SULFONANILIDE (CARONAMIDE)

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Beyer and his associates have found that 4'-carboxyphenylmethanesulfonanilide (caronamide) reversibly inhibits the excretion of the various penicillins by the renal tubules (1, 2). Excretion of p-aminohippurate by the tubules also is inhibited (3), but other renal functions including reabsorption of glucose, amino acids, urea and sulfonamide are not affected (3). It is believed that the transport mechanisms for the tubular excretion of the penicillins and p-aminohippurate are competitively inhibited by caronamide (1, 4). It was further suggested that caronamide is excreted only by filtration (1, 3, 4).

The development of methods for the estimation of caronamide in biological fluids (5) made possible the observations reported in this paper on the renal excretion and plasma binding of caronamide in the dog.

METHODS. Simultaneous creatinine and caronamide[1] clearances were performed in 3 unanaesthetized female dogs. Each dog was given 20 ml. water per kgm. body weight by stomach tube 45 minutes prior to the start of the experiments. Sufficient creatinine and caronamide were administered subcutaneously to achieve plasma levels of approximately 20 and 10 mgm per cent respectively in the plasma Urine was collected by catheter with bladder washes every 15 minutes for three consecutive periods beginning 40 minutes after the injections of creatinine and caronamide. Urine was also collected over a measured 15 minute period prior to the caronamide injection to determine the "blank" caronamide excretion. Blood was obtained at the midpoint of each period.

Bindings. The binding of caronamide on non-diffusible constituents of plasma, presumably plasma albumin, was determined by dialysis against isotonic phosphate buffer of pH 7 4 and at 37°C. for 18 hours Visking membrane was utilized for the dialysis bags. The binding of caronamide was determined on samples of plasma obtained during one of the caronamide clearance studies, and also after addition of known amounts of the drug to normal dog plasma. All bindings were done in duplicate.

Chemical methods. Creatinine was measured by a modification (6) of the Folin method. Caronamide was measured by a specific extraction method and by a non-specific precipitation method (5).

RESULTS. The overall renal clearance of caronamide in each of the 3 dogs was considerably less than that of creatinine (glomerular filtration rate), the average clearance ratios being 0.41, 0.47 and 0.62. During these experiments, the plasma caronamide levels ranged between 7.5 and 16.5 mgm. per cent, and the urine flows, between 0.15 and 5.30 ml. per minute. There was no apparent reason for the low urine flows in Dog 2. These data are summarized in Table I.

[1] The caronamide used in these studies was obtained through the courtesy of Dr. Karl H. Beyer of Sharp and Dohme, Inc., Glenolden, Pa.

' The per cent of caronamide bound on the plasma proteins varies with the concentration of the drug, ranging from 59 per cent at a plasma drug level of 13.4 mgm. per cent to 68 per cent at 3.8 mgm. per cent (Fig. 1). The binding of caronamide determined on plasma obtained from Dog 2 during the caronamide clearances (indicated by x in Fig. 1) fell, within the limits of experimental errors, on the line defining the relation between the concentration and binding of added caronamide on plasma proteins (indicated by dots in Fig. 1). The plasma binding results are all corrected to a plasma albumin content of 3.5 grams per cent, which is the approximate level found in each of the 3 experimental dogs. The albumin content in the dialysis bags in the added caronamide binding experiments was 2.8 grams per cent, the low value being the result of dilution by the added caronamide solutions. The albumin content in the binding experiment on undiluted plasma obtained from a dog with circulating caronamide was 3.5 grams per cent. The fact that the datum for the latter study (point x on Fig.

TABLE I

The renal excretion of caronamide by the dog

The clearance and ratio values are the average of 3 consecutive 15-minute periods. The clearance ratio is calculated from the whole drug clearance and the glomerular filtration rate; the excretion ratio, from the unbound drug clearance and the glomerular filtration rate.

DOG	WEIGHT	DOSE OF CARON- AMIDE	RANGE OF URINE FLOW	RANGE OF WHOLE PLASMA DRUG LEVEL	AVERAGE GLOMER- ULAR FILTRATION RATE	AVERAGE WHOLE PLASMA DRUG CLEARANCE	AVERAGE CLEARANCE RATIO	AVERAGE EXCRETION RATIO
	kgm.	grams	ml./min.	mgm.%	ml./min.	ml./min.		
1	23.6	2.00	2.85–2.95	9.3–10.8	77.3	31.3	0.41	1.07
2	16.9	2.00	0.15–0.17	13.5–16.5	54.3	25.3	0.47	1.11
3	19.1	1.25	2.20–5.30	7.5– 8.8	70.8	43.7	0.62	1.72
Averages...					33.4		0.50	1.30

1) falls on the relation, corrected to a plasma albumin content of 3.5 grams per cent, delineated by the data obtained in the added caronamide experiments, suggests that the correction for albumin concentration is valid in calculating caronamide bindings.

Since the caronamide bound on plasma protein is not available for glomerular filteration, it is necessary to correct the caronamide clearances for the binding to determine how the drug is handled by the renal tubules. The ratio of such a corrected clearence to the creatinine clearence (filtration rate) is called the excretion ratio of the drug. An excretion ratio less than one indicates that some of the filtered drug is reabsorbed by the renal tubules; a ratio greater than one indicates tubular excretion; while a ratio equal to one means that the drug is execreted by filtration only. The average execretion ratios of caronamide in the 3 dogs examined are 1.07, 1.11, and 1.72, respectively, with an overall average of 1.30 (last column, table I).

Caronamide concentrations in plasma and urine, in these experiments, were measured by both the extraction and precipitation procedures described under
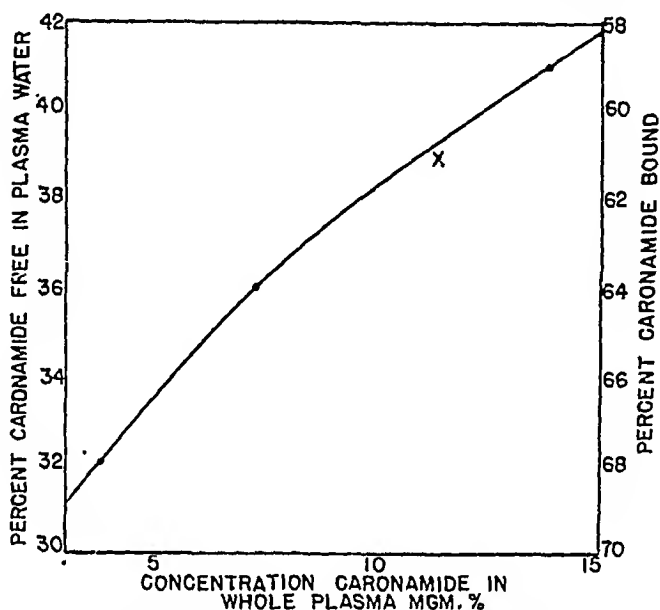
FIG. 1. BINDING OF CARONAMIDE ON NON-DIFFUSIBLE CONSTITUENTS OF DOG PLASMA

The data for caronamide added to normal dog plasma are indicated by the dots and are corrected to a plasma albumin content of 3.5 grams per cent. The cross represents the binding of caronamide determined on plasma obtained from a dog with circulating caronamide. The albumin content in this experiment was 3.5 grams per cent. All experiments were done in duplicate.

TABLE II

Caronamide metabolic product in dog plasma and urine

The values given represent the average of 3 determinations each. The extraction procedure is specific for caronamide, the precipitation procedure is not. The difference represents the metabolic product. All values were corrected for blanks.

DOG	PLASMA CARONAMIDE (MGM %)			URINE CARONAMIDE (MGM./MIN.)		
	By precipitation	By extraction	Metabolic product	By precipitation	By extraction	Metabolic product
1	12.9	9.9	3.0	4.03	3.14	0.89
2	13.6	10.8	2.8	3.69	3.86	−0.17
3	11.4	8.1	3.3	3.79	3.58	0.21

Methods. Since the extraction procedure appears to be more specific than the precipitation procedure, any difference in the drug concentrations by the two

techniques represents the presence of one or more metabolic products of caronamide. In each experiment, evidence was found for a small amount of caronamide metabolic product in the plasma (Table II). The estimation of metabolic products in the urine was complicated by the not inconsiderable urine blank of apparent caronamide in the precipitation procedure, as well as the necessity of determining the metabolic product by difference. In one dog, no evidence for metabolic product was found in the urine, while calculated overall clearances of 12 and 33 ml. per minute were observed in the other two dogs. These values are only approximations for the reasons quoted above.

To examine further the quantitative importance of the metabolic alteration of caronamide, 4.0 grams of the drug were injected intravenously into a dog. Urine was collected for 24 hours thereafter. Fifty-nine per cent was recovered as caronamide during the first 24 hours, and a negligible amount, subsequently. An appreciable amount of a more water soluble metabolic product of caronamide was also excreted by this dog over the 24-hour period.

DISCUSSION. The renal clearance of caronamide in the dog is approximately one-half the glomerular filtration rate as measured by the creatinine clearance. However, caronamide is extensively bound on plasma proteins and the calculated clearance of caronamide free in the plasma is found to be greater than the filtration rate. The average excretion ratio of 1.30 observed in the 3 dogs indicates excretion of caronamide by the renal tubules. This excreted moiety of caronamide could well account for the inhibition of penicillin and p-amino hippurate observed by Beyer and his associates (1, 2, 3).

Evidence was obtained for the presence of at least one metabolic product of caronamide in the plasmas of each dog examined. Metabolic product was demonstrated in the urine of two of these three dogs. What role, if any, that metabolic products of caronamide play in the inhibition of the excretion of penicillin and p-aminohippurate by the renal tubules, is not apparent from the data at hand.

SUMMARY

1. Forty per cent of caronamide in dog plasma is bound on plasma protein at a drug concentration of 10 mgm. per cent and an albumin content of 3.5 grams per cent.

2. The overall renal clearance of caronamide in the dog is approximately one-half the filtration rate.

3. The average ratio of the clearance of caronamide, corrected for plasma binding, to glomerular filtration rate in 3 dogs is 1.30. The excretion of caronamide, therefore, is the result both of glomerular filtration and of some renal tubular excretion.

4. Evidence is presented for the occurrence of a metabolic product of caronamide in the plasma of 3 dogs, and in the urine of 2 of the 3 dogs.

5. Fifty-nine per cent of administered caronamide was recovered in the urine in a single experiment as caronamide. There was also evidence of an appreciable amount of caronamide metabolic product in the urine.

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EFFECT OF CARBON TETRACHLORIDE LIVER DAMAGE IN THE RABBIT AND RAT ON ACETYLCHOLINE ESTERASE ACTIVITY

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Acetylcholine is hydrolyzed less rapidly by the serum of patients with liver disease than by that of normal subjects (1–6). Wescoe et al. (6) have studied the regeneration of the serum acetylcholine esterase activity in healthy subjects and in patients with liver disease after depression of the initial activity by administration of diisopropyl fluorophosphate (DFP). They found that the absolute rate of regeneration was greater in normal individuals than in patients with liver disease. A consideration of their data indicates, however, that the percentile rate of recovery was the same in the two groups. Brauer and Root (7) have reported that the acetylcholine hydrolyzing activity of rat plasma was decreased following carbon tetrachloride injury of the liver.

The original purpose of the present study was to determine, through the use of DFP, the role of the liver in the production of the plasma enzyme or enzymes which hydrolyze acetylcholine. On the basis of previous findings (8), it was believed that the administration of DFP would depress irreversibly the acetylcholine esterase activity of the plasma and liver and that the determination of the rate of regeneration of the plasma activity in normal animals and animals with various grades of liver damage would then yield data concerning the role of the liver in elaborating this enzyme. Since the blood volume of the rat was too small to allow sufficiently frequent sampling, the rabbit was chosen. I was found, however, that the acetylcholine hydrolyzing activity of the plasma of this species was not decreased in liver damage.

The objectives of this study were then re-oriented: (a) to confirm the finding of Brauer and Root that the decrease in the serum acetylcholine esterase activity of rats with carbon tetrachloride-induced liver damage was accompanied by a decrease in the corresponding liver activity; (b) to determine whether the lack of a decrease in the plasma acetylcholine esterase activity of rabbits with this type of liver damage went hand in hand with an absence of a decrease in the liver activity; (c) to determine whether the difference between the two species was due to differences, either qualitative or quantitative, in the acetylcholine hydrolyzing enzymes of plasma and liver in these two species.

EXPERIMENTAL. Mature rabbits of mixed stock and mature albino rats were used. Blood samples were drawn by heart puncture into syringes wet with heparin and the centri-

fuged plasma was used for the tests. When carbon tetrachloride was to be administered on the same day, the blood sample was taken previous to the injection of carbon tetrachloride. The livers were minced, washed free of blood, blotted with filter paper, weighed, homogenized in the Waring Blendor with four times their weight in volumes of 0 025 M sodium bicarbonate, and filtered through gauze. Such homogenates were considered a 1:5 dilution.

Enzymatic hydrolysis of acetylcholine was determined in the Barcroft-Warburg apparatus at 38°C. Three cubic centimeters of 0.02 M acetylcholine bromide freshly dissolved in 0.025 M sodium bicarbonate were placed in the main compartment of the vessel and 0 2 cc. of plasma or liver homogenate in the side arm. The final substrate concentration was, therefore, 0 0188 M acetylcholine. The gas phase consisted of 5% CO_2 and 95% N_2. The usual controls were run. The female rat plasma activity was so great under the conditions of our test that it was necessary to dilute the plasma 1:3 with 0 025 M sodium bicarbonate to obtain a conveniently measurable rate of gas production. Acetyl-β-methylcholine and benzoylcholine were also used, as described later in the text.

Carbon tetrachloride was injected intraperitoneally without diluent

The bromsulphalein (BSP) liver function test was used in rabbits. Ten minutes after an injection of 10 mg. per kgm. of BSP into the ear vein a heparinized blood sample was removed from the heart. Plasma was obtained and diluted 1:10 with 0.3% sodium hydroxide solution. This treated plasma was compared visually with standards which contained known concentrations of bromsulphalein The 100% standard was a solution containing 0 1 mg. per cc. of BSP diluted 1:10 with 0.3% NaOH. The values found for retention of the dye in normal rabbits was zero to 20%

RESULTS. *The normal range of plasma acetylcholine esterase activity in the rabbit and the rat.* It was necessary to determine the daily variability of the plasma acetylcholine esterase activity in normal rabbits and rats in order to assess reliably the effect of carbon tetrachloride induced liver damage on the activity of this enzyme. Blood, for determination of plasma acetylcholine esterase activity, was taken from various members of a control group of 12 rabbits at intervals of 1 to 2 days for a period of 12 days. Although there were fluctuations in enzyme activity, there was no trend which could be attributed to repeated bleeding, maintenance under laboratory conditions, etc. The level of activity bore no relation to sex, as has been reported for the rat (9 to 12). In 40 determinations, the activities ranged from 49 to 87 and averaged 70 cu. mm. CO_2 per 0.2 cc. plasma per hour. Similarly, 17 determinations made in a group of 7 male rats during a period of 9 days showed values ranging from 66 to 154 and averaging 96 cu. mm. CO_2 per 0.2 cc. plasma per hour. Six determinations in 3 female rats showed distinctly higher values, ranging from 200 to 522 and averaging 372 cu. mm. CO_2 per 0.2 cc. plasma per hour.

Liver damage and plasma acetylcholine esterase. Groups of rabbits and rats were injected intraperitoneally with various amounts of undiluted carbon tetrachloride. Most of the animals were given daily doses but a few were given injections at longer intervals as noted in the tables. Both rabbits and rats when treated with carbon tetrachloride lost weight. The administration of carbon tetrachloride caused a 30 to 100 per cent retention of bromsulphalein in the rabbit. Histological study of representative sections of several rabbit and rat livers showed marked damage due to treatment with this hepatotoxin.[3]

[3] We are indebted to Dr. T. Harvey of the Pathology Section of this laboratory for the pathological reports.

Table 1 shows that doses ranging from 0.05 to 0.5 cc. carbon tetrachloride per kgm. administered daily for periods up to 12 days did not significantly alter the plasma acetylcholine esterase activity in the rabbit. It may also be seen that higher daily doses, 0.5 cc. per kgm., for periods of 3 days and, in two other instances, of 5 to 7 days, did not seem to change this plasma enzyme activity. The average value of 41 determinations in 18 rabbits at various periods after the beginning of carbon tetrachloride injections was 67 cu. mm. CO_2 and was not

TABLE 1

Effect of intraperitoneal injections of carbon tetrachloride on plasma acetylcholine esterase activity in rabbits

Results are expressed as cu. mm. CO_2 liberated by 0.2 cc. plasma per hour from the standard reaction mixture.

RABBIT NO.	DAILY DOSE	ACTIVITY BEFORE CCl4	ACTIVITY AFTER BEGINNING OF CCl4 ADMINISTRATION ON DAY								
			2	3	4	5	7	9	11	12	15
	cc. per kgm.										
864	0.05	53			42				38		44
868	0.05	66			53				43		56
856	0.1	74		71	78				76		83
857	0.1	62			67				54		54
858	0.1	66			53						59
852	0.2	86		83	83						
853	0.2	65		54	54						
854	0.2	61		52	59						
207	0.2	67	64								
208	0.2	73	80			56					
209	0.2	81	74			64		66		91	
210	0.2	83	72			71		87		113	
201	0.5	66	70			53					
202	0.5	78	95								
203	0.5	74	86	99							
991	0.5	102		51							
994	0.5	56									
910	0.5	77		81							

Average activity in rabbits was 72 cu. mm. CO_2 per hour before administration of CCl_4, and 67 cu. mm. CO_2 per hour for the CCl_4 period. The difference between the two was not significant.

significantly different from the average value, 72 cu. mm. CO_2, of the determinations before carbon tetrachloride administration.

In contrast, as may be seen from table 2, the administration of carbon tetrachloride to rats led to significant decreases in the plasma acetylcholine esterase activity. The average value of the activity of normal plasma in 11 male rats was 110 cu. mm. CO_2 per 0.2 cc. plasma per hour. The average value of 24 determinations at varying intervals after the beginning of the carbon tetrachloride administration was 90 cu. mm. CO_2 per 0.2 cc. plasma per hour. This difference was barely significant ($t = 2.38$; $p = 0.02$). In the female rats a marked de-

crease from an average control value of 714 to one of 137 cu. mm. CO_2 per 0.2 cc. plasma per hour was obtained on the eighth day following beginning of administration. This decrease was highly significant (t = 8.3; p = <0.01).

Acetylcholine esterase activity of the livers of untreated and carbon tetrachloride treated rabbits and rats. If the acetylcholine esterase activity of the plasma is due to the formation of the corresponding enzyme or enzymes in the liver, it

TABLE 2

Effect of intraperitoneal injections of carbon tetrachloride on plasma acetylcholine esterase activity in rats

Rats No. 1–6 received 0 2 cc. per kgm daily. Rats *No.* 8–16 received 0.5 cc. per kgm. on the 3rd, 5th, and 7th days after the control enzyme determinations. Results are expressed as cu. mm. CO_2 liberated by 0 2 cc plasma per hour from the standard reaction mixture

RAT NO	ACTIVITY BEFORE CCl4	ACTIVITY AFTER BEGINNING OF CCl4 ADMINISTRATION ON DAY					
		4	6	7	8	9	11
a. Male rats							
1	95	69		54		67	94
2	139	122		88		107	88
3	118	116		77		81	
4	126	111		74			
5	109	92		91		103	101
6	88		76				
7	111		107				
8	154				97		
9	129				75		
10	85				96		
11	50				62		
b. Female rats							
12	627				165		
13	575				162		
14	738				80		
15	956				92		
16	676				187		

Average activity in male rats was 110 before administration, 90 for the CCl4 period. The difference is barely significant (t = 2 38, p = 0 02). Average activities in female rats for control and CCl4 periods were respectively, 714 and 137, the difference was highly significant (t = 8; p = <0 01).

may be expected that, following the administration of carbon tetrachloride, the enzyme activity of the liver of each species would parallel that in its plasma Table 3 shows that no significant alteration in the acetylcholine esterase activity of the rabbit liver could be demonstrated following the daily administration of 0.2 cc. per kgm. of carbon tetrachloride, although histological examination of rabbit liver sections showed severe damage. Another group of rabbits (not shown in table 3) was given a larger dose of carbon tetrachloride, but at less fre-

quent intervals. These were injected intraperitoneally with 0.5 cc. of CCl$_4$ per kgm. on days 0, 2 and 5. Animals which died or were sacrificed on the stated day showed the following activity of liver esterase: 2nd day—147, 36 and 43; 6th day—42 and 34; 7th day—36 and 85 cu. mm. CO$_2$. Again, no significant alteration from the normal rabbit liver esterase activity was demonstrable.

On the other hand, table 3 shows, as Brauer and Root (7) have previously

TABLE 3

Acetylcholine esterase activity of the livers of normal and carbon tetrachloride treated animals

The animals were injected with 0.2 cc. CCl$_4$ per kgm. daily for the periods shown. The liver enzyme activity is expressed as the cu. mm. CO$_2$ per hour liberated by 0.2 cc. of a 1:5 liver homogenate in 0.025 NaHCO$_3$ from the standard reaction mixture.

ACTIVITY BEFORE CCl$_4$ ADMINISTRATION	ACTIVITY AFTER BEGINNING OF CCl$_4$ ADMINISTRATION ON DAY			
	2	3	5	6
a. Rabbits				
56		94		95
65		60		57
67		37		71
108		58		
46				
110		49		
76				
b. Male rats				
49	15		18	
56	14		29	
62	13		40	
c. Female rats				
114	50		56	
189	41			
252	42			

The average enzyme activities of rabbit liver before and after CCl$_4$ administration were, respectively, 75 and 65 cu. mm. per hour. The difference was not significant (t = 0.88; p = 0.4). The average enzyme activities of male rat liver plasma before and after CCl$_4$ administration were, respectively, 56 and 21 cu. mm per hour, and of the female rat liver, 185 and 47 cu. mm per hour. These differences were definitely significant.

found, that the rat liver acetylcholine esterase activity undergoes a marked decrease following carbon tetrachloride injury. In the male rats, the activity decreased from an average value of 56 to one of 21 cu. mm. (t = 12; p = <0.01). In the female rats, the enzyme activity decreased from 185 to 47 cu. mm. (t = 34; p = <0.01). Both decreases were highly significant.

The finding that the liver and plasma acetylcholine esterase activities of the rabbit were not changed after carbon tetrachloride administration, whereas

those of the rat were decreased, raised the possibility that the enzyme or enzymes responsible for these activities differed in the two species. It has been shown (8) that DFP and physostigmine inhibit rabbit serum acetylcholine esterase activity to a different degree from that of other species. Sawyer (13) has presented data indicating that the acetylcholine esterase activity of the rabbit liver differs from that of rat liver. In order to explore the species difference noted in the present experiments, the relative rates of hydrolysis of acetyl-β-methylcholine, acetylcholine and benzoylcholine were studied.

The concentration in the final reaction mixtures were 0 0188 M for the first two of these substrates and 0.0094 M for benzoylcholine. It was found, in

TABLE 4

The comparative rates of hydrolysis of acetyl-B-methylcholine, benzoylcholine and acetylcholine by the plasma and liver of rats and rabbits

	RATIO OF RATES OF HYDROLYSIS					
	Acetyl B methylcholine/acetylcholine			Benzoylcholine/acetylcholine		
	No of detns	Average	Range	No of detns	Average	Range
a Rabbits						
Plasma	19	0 59	0 48-0 81	5	0.81	0 06-1 5
Liver	7	0 94	0.88-0 99	6	0.78	0 06-1 8
b Male rats						
Plasma	6	0.34	0 25-0 42	2	0.18	0.15-0 20
Liver	5	0 12	0 11-0 13	3	0 22	0.11-0 30
c Female rats						
Plasma	6	0 07	0 05-0 09	3	0 14	0 07-0 19
Liver	3	0 08	0 06-0 10			

confirmation of Mendel, Mundell and Rudney (14), that these concentrations of acetyl-β-methylcholine and benzoylcholine gave maximal action and that higher concentrations of these substrates did not inhibit the activity. A concentration of 0.0188 M acetylcholine yielded maximal activity with rat plasma and optimal activity with rabbit plasma.

It may be seen from table 4 that, for rabbit plasma, the average ratio of the rate of hydrolysis of acetyl-β-methycholine to that of acetylcholine (0 59) approximates that for rabbit liver (0 94) more closely than those for male and female rat plasma (0 34, 0 07) or rat liver (0.12, 0.08)   Conversely, the ratios of the hydrolysis of these two substrates for male rat plasma (0 34) and liver (0 12) are much more within each other's range than within the range of values for rabbit plasma and liver. The same holds for female rat plasma and liver.

The ratios of the rates of hydrolysis of benzoylcholine and acetylcholine vary much more widely but, in general, the averages as well as the individual values for rabbit plasma and liver approximate each other and the averages are distinctly higher than the average values for rat plasma and liver.

Discussion. The present work has shown that carbon tetrachloride damage to the liver in rats is accompanied by significant decreases in the acetylcholine esterase activities of the liver and plasma. These results confirm the findings of Brauer and Root (7). Our results show, however, that in the rabbit carbon tetrachloride damage to the liver, although manifested by marked histological change and bromsulfalein retention, does not bring about a significant decrease in the acetylcholine esterase activity of the liver or plasma.

This species difference in susceptibility of the liver acetylcholine esterase activity to decrease by carbon tetrachloride damage may be explained in either of two ways: (a) the enzyme in the rabbit liver cells is not accessible to action by the carbon tetrachloride; (b) the rabbit liver enzyme differs from the rat liver enzyme. There is no evidence available either to support or deny the first possibility.

The second possibility gains considerable support from previous reports in the literature as well as the present work. Mendel and his co-workers (14) showed that the ratio of hydrolysis of acetyl-β-methylcholine to that of acetylcholine by those cholinesterases which he designated as "true" was about 0.85. The present work yields an average value of 0.94 for the ratio for rabbit liver and a distinctly different average value, 0.12, for the rat liver. Again, Mendel and others (14–16) have pointed out that another group of cholinesterases designated by them as "pseudo", yields a ratio of 0.25 for the rate of hydrolysis of benzoylcholine to that of acetylcholine. The present results show that rat cholinesterase falls into this group, for the average value of the ratio of hydrolysis of these two substrates is 0.22, whereas the value for the average ratio for rabbit liver cholinesterase is 0.78.[4]

It is also of interest that, in the rat, the decrease of the liver acetylcholine esterase activity was accompanied by a decreased plasma activity, and that, in the rabbit, absence of a decrease in the liver acetylcholine esterase activity went hand in hand with an absence of a decrease in the plasma. If it is assumed, as has been indicated by previous work (8), that the liver is the source of the plasma acetylcholine esterase, then any agent inactivating the liver cholinesterase should result in a decrease of the plasma cholinesterase. On the other hand, any hepatotoxic agent which does not affect the liver cholinesterase should, similarly, be without effect on the plasma cholinesterase. Our results are in accord with this formulation. Moreover, the plasma cholinesterase, in both the rabbit and the rat, resembles the liver cholinesterase with respect to the acetyl-β-methyl/-acetylcholine and benzoylcholine/acetylcholine ratios. These findings also indicate that the plasma acetylcholine esterase is derived from the liver.

[4] Mendel's classification of cholinesterases into 'pseudo' and 'true' types has been critically reviewed by one of the authors elsewhere (17).

SUMMARY

1. Liver damage resulting from the administration of carbon tetrachloride does not reduce the acetylcholine esterase activity of the plasma or liver of the rabbit. We have confirmed a previous report that in the rat plasma and liver activities are decreased in liver damage due to carbon tetrachloride.

2. This species difference is attributed to the differences in the choline ester hydrolyzing enzymes found to be present in the plasma and liver of the two animal species.

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4'-CARBOXYPHENYLMETHANESULFONANILIDE (CARONAMIDE): ITS TOXICOLOGIC EFFECTS

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A series of reports relating to caronamide has shown it to inhibit the renal tubular mechanism responsible for the excretion of penicillin (1) without influencing the other renal transport mechanisms tested (2). Its mode of action is to inhibit the penicillin transport mechanism itself in contrast to PAH (p-aminohippurate) which, like penicillin, is excreted by the tubules and so produces its effect through a "mass action" type of inhibition (3). Thus caronamide makes possible at low dosages a considerable physiologic economy of penicillin that results in a manifold increase in antibiotic blood level and an attending increase in its therapeutic efficacy during a given dosage regime (4).

Caronamide was synthesized in the Department of Organic Chemistry of these laboratories and has been described by Sprague, Ziegler, Miller and Cragoe (5). Its chemical name is 4'carboxyphenylmethanesulfonanilide and its structural formula is as follows:
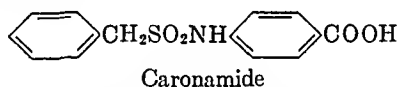
Caronamide

The purpose of the work leading to this report was the evaluation of the acute and chronic toxicity of caronamide. Such a study assumes considerable importance in the overall consideration of the compound. Any agent that is to be used with penicillin should have a low order of toxicity so as not to limit the usefulness of the antibiotic agent itself in such a combination.

ACUTE TOXICITY STUDIES. *Mice.* We customarily use the mouse intravenous LD_{50} as an indication of the inherent toxicity of a compound, especially from a comparative standpoint. Also, in this work we have obtained data on oral and subcutaneous acute toxicity. A comparison of the toxicity data obtained by these three modes of administration is useful in indicating the magnitude of oral adsorption.

Figure 1 represents a series of dosage response curves for the intravenous, subcutaneous, and oral toxicity of caronamide. In these experiments the compound was administered as a 10 per cent solution of its sodium salt, pH adjusted to neutrality. The coordinates for the curves are represented by data on 50 mice per dosage level for the intravenous toxicity curve and 10 animals per dosage level for the subcutaneous and oral administration. Carworth CF_1, female mice weighing 18-20 grams were used in this study. It may be seen that the compound is of a low order of toxicity, the figures for LD_{50} being 1405 ± 40 mg/kg when administered intravenously, 1650 ± 103 mg./kg. when given subcutaneously,

and 2450 ± 219 mg./kg. when administered orally. The spread of the data and the slope of the curve tend to indicate only a moderate variation in response within the species.

Regardless of the mode of administration, the mice given lethal doses became lethargic 15 to 30 minutes after receiving the compound. This lethargy gradually deepened until death occurred 2 to 4 hours later. There were a few deaths at 12 hours when the drug was administered orally; otherwise, there were no late or delayed deaths. When given orally or subcutaneously the lethargic state was preceeded by excitation as manifested by running and jumping. Death appeared to be caused by respiratory failure.
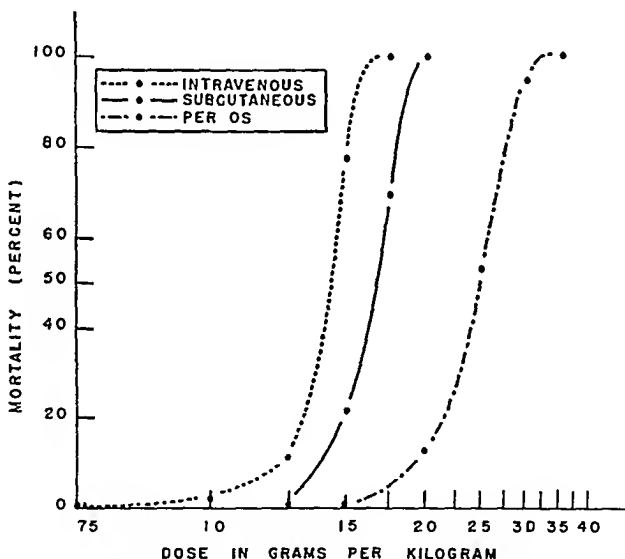
FIG. 1. DOSAGE-MORTALITY CURVES FOR CARONAMIDE ADMINISTERED TO MICE INTRAVENOUSLY, SUBCUTANEOUSLY AND ORALLY
(Behrens, D.: Arch Exp. Pathol Pharmacol , 140: 237, 1929)

The intravenous LD_{50} for caronamide is sufficiently reproducible to be useful for biological assay of various lots of the compound. The data from assays of six tests on the compound are presented in table 1. The first 4 lots were prepared in the laboratory and the last two tests were run on a large pilot plant batch of material. Using the figure for LD_{50} of the first lot as standard, only the fifth assay was sufficiently low to have a significant t value. Although the mice in this test were from the same source and strain as the others they were obtained from another department in the Research Division for this purpose. Differences in room temperature and in handling mice in the two departments may have been responsible partially for the variation in the data, since in the last test mice of the same source, weight, and strain but housed in our quarters were used.

One last point of comparison has been made in mice between the toxicity of caronamide and the potassium and sodium salts of crystalline penicillin G. The penicillin salt was weighed accurately and made up in aqueous solution. An aliquot was submitted for assay and the remainder was used immediately. The order of injecting the dosage increments was randomized to avoid any effect of decreasing penicillin potency on the data. The solutions assayed 108 ± 7.03 per cent of theoretical potency by the high-low Florey cup plate method.

TABLE 1

A series of acute intravenous caronamide toxicity data for mice

Each letter represents a separate sample of caronamide. The numerals indicate two assays on a single sample of drug wherein mice from different laboratories were used.

SAMPLE	TOXICITY, MG./KG.		
	LD_0	$LD_{50}*$	LD_{100}
B	750	1425 ± 85	1750
M	1000	1325 ± 105	1750
N	1250	1450 ± 70	1750
Q	1250	1450 ± 71	1750
1†	1000	1300 ± 88	1750
2	1000	1375 ± 68	1750

Cumulative $LD_{50} = 1405 \pm 40$ mg./kg.

* Calculated by the method of Epstein, B. and C. W. Churchman: Ann. Math. Stat., 15: 90, 1944.

† Omitted from the cumulative LD_{50} and the calculations for figure 1.

TABLE 2

The comparative acute intravenous toxicity of the sodium and potassium salts of crystalline penicillin G administered to mice

Dosage is expressed in terms of penicillin or caronamide *per se*

MORTALITY	POTASSIUM PENICILLIN G*	CARONAMIDE	SODIUM PENICILLIN G†
	mg./kg.	*mg./kg.*	*mg./kg.*
LD_0	392	750	2049
LD_{50}	564	1405	3090
LD_{100}	784	1750	4146

* Calculated on a basis of 1595 units/mg.

† Calculated on a basis of 1628 units/mg.

Since this is within the acknowledged error of the assay, no correction has been introduced into the toxicity data.

From the data presented in table 2 it may be concluded that the intravenous toxicity of caronamide in mice is intermediate between that of the potassium salt and the sodium salt of crystalline penicillin G, on a mg./kg. basis. We do not mean to imply that the margin of safety or therapeutic ratio for caronamide is as great as for these salts of penicillin, for such is not the case. The dosage of caronamide is in terms of grams per day whereas that for penicillin is in milligrams or Oxford units.

Rabbit. Adult, albino rabbits in good health and weighing 2 kilograms or more were given intravenously dosage increments of sodium caronamide as a 10 per cent aqueous solution. The dosage was calculated in terms of the acid and was infused by gravity drip, using a 0.2 cc. graduated burette as the reservoir. The rate of infusion was 1 cc./min.

The numerical results of these tests are included in table 3. It may be seen that the increment between the LD_0 and LD_{100} is not great. The LD_{50} calculated by the method of Kärber was 1.32 gm./kg.

No abnormal symptoms were noted during the infusion period. When removed from the table at the completion of the infusion the animals had use of their righting reflex, but some lack of coordination, particularly of the hind limbs,

TABLE 3

The acute intravenous toxicity of caronamide (calculated as the acid) administered to rabbits and to dogs

DOSE	NO OF ANIMALS	NO OF DEATHS WITHIN			TOTAL DEATHS	LD_{50}*
		4 hr.	8 hr.	Later		
Rabbits						
gm/kg						
0 5	1	0	0	0	0	1 32
1 0	5	0	0	0	0	gm./kg.
1.25	5	0	1	0	1	
1.35	5	3	2		5	
1.50	5	1	4		5	
Dogs						
0.5	1	0	0	0	0	1 575
1.0	1	0	0	0	0	gm /kg.
1.25	5	0	0	0	0	
1.50	5	0	2	0	2	
1.75	5	3	1	0	4	
2 00	5	2	3		5	

* Kärber. G Arch Exp Pathol and Pharmacol, **162**: 480, 1931

was noted. The iris was markedly constricted (pinpoint pupil), the cornea was cloudy, and the vessels of the sclera and sclerocorneal junction were injected.

The majority of the rabbits became lethargic. Recovery or death occurred within 4 to 8 hours after completion of the infusion. Marked lethargy preceeded death. Death was attributed to respiratory failure. The heart continued to beat for several minutes after all apparent respiratory movements had ceased.

Dog. Twenty-two apparently normal, adult mongrel dogs weighing 8 kg. or more were used in this study. A 10 per cent soluton of caronamide (pH 7.8) was infused intravenously at a rate of 1.0 to 1.5 cc./min. by means of gravity drip, using a burette graduated in 0.2 cc. as a reservoir.

The intravenous LD_{50} of caronamide for dogs was 1.575 gm./kg. when cal-

culated by the method of Kärber. The dosage response data are summarized in table 3.

The symptomatology of toxicity was qualitatively similar in all the animals, the severity of the manifestations being related to dosage. During the infusion period most of the dogs became nauseated. This was followed by vomiting and defecation. Dilation of the vessels of the sclera, nictitating membrane and conjunctiva, myosis and photodysphoria were noted. As the infusion progressed the orbit of the eye tended to recede into its socket and it rotated upward and outward slightly as is seen commonly in anesthesia. Dryness of the oral mucous membrane was observed.

Within an hour after completion of the infusion the dogs became mildly sedated and slept when undisturbed. The dogs that did not die recovered from their lethargy within four to eight hours following its onset. Those that did not recover from their drowsiness died within three to twelve hours. Death was preceded by hypnosis, tonic convulsions, shallow rapid breathing, and finally, cessation of respiration. The heart continued to beat for several minutes after all apparent respiratory movements had ceased.

CHRONIC TOXICITY STUDIES. *Rat.* Twenty-three normal female albino rats weighing between 125 and 150 grams were selected for the experiment. Each was given 0.54 cc. of water twice daily for a control period of two weeks. During this time control hematologic studies were performed at weekly intervals.

Following the control phase the rats were divided into two groups of equal weight, one group being designated as controls, the other as test animals. For three months thereafter the test rats were given a solution of caronamide by stomach tube twice a day five days a week and once on Saturdays. The total daily drug dosage was 0.5 gm./kg., the average volume of solution per dose being 0.54 cc. At the same time the control rats were intubated with 0.54 cc. of water. In the course of the experiment three test animals and one control rat died; one rat in each group was sacrificed because of intercurrent infections.

Weight change, hemoglobin, erythrocyte and leucocyte count, hematocrit, and differential counts were recorded weekly during the control phase and the first three weeks of the experimental portion, and every two to three weeks thereafter.

The results of the experiment, as given in table 4, indicate that there was no difference in the hematologic picture of the two groups of rats. Whereas the rats given the drug maintained their weight, the control animals gained 8 grams in three months. The difference in the differential white cell counts in the latter part of the experiment are attributable to a change in personnel reading the slides instead of any sudden change in the experiment.

At the termination of the experiment the rats were sacrificed and sections of the following organs were taken for histologic examination: lymph node, nerve, thymus, trachea, lung, heart, esophagus, stomach, small intestine, large intestine, pancreas, spleen, adrenal, liver, kidney, ovary, uterus, and skeletal muscle. Histologic examination of these organs in our laboratory and by a consultant pathologist revealed no differences between the control rats and those receiving 0.5 gm./kg. of caronamide per day.

Dogs and monkey. Eight dogs were used for this work. Unfortunately only one Rhesus monkey was available or obtainable for the study. The dogs were

TABLE 4

Results of a chronic toxicity experiment wherein the test rats were given 0.5 gm. of caronamide/ kg./day in solution by stomach tube

The control rats were intubated with an equivalent amount of water. Values represented are averages for each group at a given date.

DATE	GROUP	WT.	WBC	RBC	Hb	HEMAT.	DIFFERENTIAL*				
							N	L	M	E	B
Orientation Period											
		gm.	thous.	millions	gm.	%					
6–19	Control		10.9	9.9	18.1	49	25.1	72.8	1.5	0.9	0
	Test		14.0	10.6	18.2	55	21.5	74.7	1.6	2.4	0
6–26	Control		9.1	9.1	17.9	51	22.1	74.4	0.9	2.0	0.125
	Test		12.6	9.0	18.2	48	25.3	71.2	1.6	2.0	0
Toxicity Study											
7–2	Control	215									
	Test	215									
7–9	Control	214	11.3	9.0	17.2	48	25.0	71.0	0.9	3.0	0
	Test	210	12.1	9.1	16.8	50	25.3	70.2	0.9	3.6	0
7–16	Control	218	10.5	8.5	16.1	45	24.4	71.0	1.8	2.9	0
	Test	216	13.3	8.6	16.0	46	27.2	67.7	2.0	3.1	0
7–23	Control	211	10.0	8.4	17.8	48	25 1	70.1	1.9	3.1	0
	Test	212	11.1	8.1	17.9	47	21.7	75.5	0.9	2.2	0
8–13	Control	226	10.0	8.5	18.0	49	25.1	70.8	1.9	2.6	0
	Test	216	10.5	8.6	17.6	50	23.7	74.8	0.6	1 0	0
8–27	Control	225	11.5	8.7	16.7	48	27.1	57.6	12.8	2.4	0
	Test	217	8.5	7.7	18.5	48	28.7	56.3	12.5	2.2	0.05
9–11	Control	224	11.7	8 3	18.6	51	22.5	59.1	15.1	2.8	0.125
	Test	217	11.1	8.0	18.1	52	23.5	60.1	14.3	2.1	0
	Control	224	13.9	7.7	16.3	53	26.8	49.3	20.0	3.6	0.125
	Test	216	15.1	7.4	16.8	53	26.5	53.8	18.1	1.6	0.111

* N—Neutrophils, L—lymphocytes, M—monocytes, E—eosinophils, B—basophils. Expressed in per cent.

all beagles, raised in our kennels and, except for one, were either litter mates or of about the same age. They were approximately 12 months old.

The design of the experiment included two control dogs and two animals on

each of three oral dosage increments: 0.4 gm./kg./day, 1.0 gm./kg./day, and 1.5 gm./kg./day. The total daily dosage was divided into 6 aliquots, and was administered as the sodium salt in about 100 cc. of water by stomach tube at 4-hour intervals day and night. The control dogs received water at the time the drug was given to the other animals.

The dogs were weighed, placed in metabolism cages so that 24-hour urine collection could be obtained, changed from a meat and pellet diet to one of Friskies Dog Pellets fed twice a day, and were allowed a three to five day equilibration period before beginning administration of the drug. Daily throughout the initial period oxalated blood samples were obtained for chemical analysis. Other blood samples were taken for hematology, and 24-hour urine collections were obtained. After the drug phase was begun the blood and urinalyses were performed at least twice a week for the duration of the four weeks. Supportive vitamin therapy was given throughout the experiment.

Records were kept of the dogs' weight, appetite, and general physical condition. The hematology consisted of erythrocyte and leucocyte counts, white cell differentials, hemoglobin, and hemotrocrit. Blood chemistry consisted of estimations of plasma creatinine, urea N, NPN, and, in some cases, glucose. Urinalysis included total 24-hour output, qualitative albuminuria, qualitative and quantitative reducing substance, pH, specific gravity, creatinine, urea N, and total nitrogen. Studies on the monkey were limited to hematology. It received 1.0 gm. of drug/kg./day by stomach tube on the same dosage schedule as for the dogs.

At the termination of the experiment the animals were given a final physical examination, sacrificed under barbiturate anesthesia by exsanguination, and were autopsied immediately. Sections were taken for histologic study in our laboratory and by a consulting pathologist. Tissues taken for examination included the various lobes of both lungs, heart, sections from the lobes of the liver, upper, middle and lower regions of both kidneys, adrenals, spleen, pancreas, stomach, small intestine, colon, cervical and abdominal lymph nodes, pituitary, ovary or testis, skeletal muscle, motor and medullary areas of brain, spinal cord, thyroid, gall bladder, ureter, urinary bladder, trachea, esophagus, and pancreas.

Results: General appearance of all the dogs was good throughout the test. They were alert, responsive, and friendly. At the termination of the study the dogs and monkey were in good health, ate well, their coats glistened, their reflexes were normal, and their muscle tone and gait were normal. The greatest loss of weight was a little over one kilogram, although some dogs lost little or no weight and the distribution of weight loss was not uniformly related to dosage.

Hematology. It would be difficult to say with assurance that there was any effect of the compound at any dosage on the peripheral blood picture of these dogs and the monkey, figures 2 through 10[1]. Certainly one did not observe the profound leucopenia and anemia that would be characteristic if sulfonamides were administered under these conditions. Dogs receiving oral dosages of 1.5 gm./kg. of most well-absorbed sulfonamides per day could not be expected to survive this test.

It may be pointed out that toward the end of the study both dogs on the 1.5 gm./kg./day dose showed a marked neutrophilic leucocytosis with a decrease in hemoglobin and a relative fall in lymphocytes. However, one cannot be certain that the onset of estrous in dog 202 was not responsible or did not contribute in large measure to these effects. The fact that a similar picture occurred in dog 203 on the 1.5 gm./kg./day dose does not alter this interpretation because, as evidenced by their numbers, the dogs were litter mates and females. Thus it would appear likely that the second dog soon obviously would have been in heat. The changes in hematology in the other dogs receiving the drug were no greater or more predictable than in the controls.

Although the monkey developed a respiratory infection during the experiment it recovered without therapy and appeared in good health at the end of over 5 weeks. Its hematologic picture did not indicate any deleterious effects of the drug.

Blood and urinalysis. These data have been presented in the form of curves in figures 11 through 18[1]. Unfortunately, insufficient time was permitted during the control phase of the tests to permit the plasma and urine levels of metabolites to equilibrate. Indeed it can be said for the biproducts of protein metabolism that there was considerably greater difference in changing from a meat and pellets diet to an all-pellet regime than followed administration of the drug. This is most apparent in the dogs receiving the high drug dosage: their equilibration period before blood and urine samples were taken was shorter than for the control dogs and those on the lower drug dosage. There appears to be no consistent effects of caronamide on the blood or urine level of protein metabolites.

Urinary volume was influenced for the most part by the amount of water administered with the drug. This deliberately was kept up so as to avoid the state of partial dehydration seen in animals not accustomed to close confinement, as reflected in the urine volumes for some of the dogs during their control phase. In general the pH of the urine of both control and test dogs became more alkaline during the experiment. This may have been due to the change in their diet. The compound did not produce proteinuria. It is necessary to avoid strong acids in testing for proteinuria, for caronamide is precipitated from strongly acidic urine, and so may give a falsely positive test if the analyst is not aware of this chemical characteristic of the compound.

The only intersting metabolic aberration induced by caronamide was the appearance of reducing substance in the urine of dogs on the 1.0 gm./kg./day and 1.5 gm./kg./day dosage regimes. At first our concern was that the compound might be inhibiting the reabsorption of glucose or causing a hyperglycemia. Neither interpretation appears to be true.

Caronamide neither altered the blood sugar level of these dogs nor decreased

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[1] The illustrations referred to herein as figures 2 through 18, and which include comprehensive hematologic and biochemical data on each animal, were submitted to the editor of this journal together with the manuscript. They have not been published in the interest of economy of space, but the authors have the permission of the editor to supply the additional illustrations for a limited time with reprints to individuals requesting them.

glucose Tm as determined by suitable renal function studies on other dogs (2). It was found that when yeast was added to urine the reducing substance was not fermented, for the presence of the reducing substance remained in the urine in the same amount as before yeasting. Glucose added to urine was fermented under these conditions. Tests for glucuronates were positive occasionally. Tests for lactose were negative.

The results suggest, but do not prove, that the reducing substance may be a pentose. We have been unsuccessful in obtaining reliable osazone crystals for identification of the reducing substance in the urine and so have no conclusive proof of identity of the substance. It is interesting in this connection that some children under the stress of infection infrequently and unpredictably excrete a non-fermentable reducing sutstance in their urine when they receive 0.4 gm./kg./day of caronamide in divided doses at 4 hour intervals (6). It is of course possible that the reducing substance is a metabolic product of caronamide itself.

Pathology. Examination of sections taken from the various organs and prepared for histologic study using various staining procedures revealed no definite evidence of tissue damage attributable to the drug at any dosage. This impression was confirmed after inspection of the slides by a consultant pathologist.

SUMMARY

The order of acute intravenous toxicity of caronamide, as represented by the LD_{50}, was quite comparable for mice, rabbits and dogs; being 1405 mg./kg., 1320 mg./kg. and 1575 mg./kg., respectively. The magnitude of the toxicity in mice was intermediate between that for crystalline potassium penicillin and sodium penicillin. The principal manifestation of toxicity was the sedation that the compound seemed to produce in all three species of animals at high dosages.

The oral administration to rats of 0.5 gm. of caronamide/day seemed to produce no evidence of hematologic or histologic damage as compared to control animals. Similarly, dogs were given dosages of from 0.4 to 1.5 gm./kg./day for a period of four weeks, and a monkey was given 1.0 gm./kg./day for four weeks. Of the studies involving blood and urine chemistry, hematology and histology, a non-fermentable reducing substance which appeared in the urine of dogs receiving the higher drug dosages was the only definite manifestation of a systemic effect attributable to the drug.

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CERTAIN PHARMACOLOGIC PROPERTIES OF 4'-CARBOXYPHENYLMETHANESULFONANILIDE (CARONAMIDE), INCLUDING ITS EFFECT ON THE RENAL CLEARANCE OF COMPOUNDS OTHER THAN PENICILLIN

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Sprague, Ziegler, Miller, and Cragoe have described a compound (1) that has been found to increase substantially penicillin blood level response by decreasing the renal excretion of penicillin (2). It has been proposed that the mode of action of the compound, (4'-carboxyphenylmethanesulfonanilide, caronamide), is by halting or slowing the transport mechanism responsible for the tubular excretion of penicillin (3). This would be in contrast to the mode of action of PAH (p-aminohippurate) which does not suppress the function of that transport system but in being excreted by that mechanism limits its functional capacity to excrete penicillin (4) The purpose of this report is to present certain pharmacologic properties of caronamide including its effect on the renal clearance of compounds other than penicillin.

PHYSICAL AND CHEMICAL CHARACTERISTICS OF CARONAMIDE The compound is a colorless crystalline substance. The acid has little or no taste or odor As an acid it is only very slightly soluble in water or such organic solvents as carbon tetrachloride, chloroform, or benzene. It is fairly soluble in ethyl alcohol, acetone, and propylene glycol Both the mono and di sodium salt are very soluble in water. Thus solutions of the compound can be prepared by dissolving the acid in an equivalent amount of sodium carbonate or sodium hydroxide solution. It is relatively stable to hydrolysis with acid or base.

The structural formula for the compound is:

$$\text{\langle\hspace{-0.3em}\rangle CH}_2\text{SO}_2\text{NH\langle\hspace{-0.3em}\rangle COOH}$$
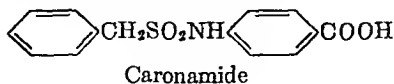
Caronamide

The molecular weight of the acid is 291. Its uncorrected melting point is 229–230°C

THE ANALYTICAL DETERMINATION OF CARONAMIDE IN PLASMA AND URINE We have used three methods for the determination of caronamide in plasma and urine These methods differ somewhat in their reproducibility and specificity for the compound

The first method available to us was *the dialysis spectrophotometric* method for carona mide in plasma which was developed with the cooperation of Mr. Joseph L Cimmera of the Department of Pharmaceutical Chemistry. This method is based on dialysis of the compound from diluted plasma into 0 1 N sodium hydroxide When the pure compound is dissolved in 0 1 N sodium hydroxide its absorption peak as determined with the aid of a Beckman spectrophotometer is 280–281 mµ The extinction coefficient is 71 2 for the pure solution and 68 7 when dialyzed from plasma

In this procedure 0.5 cc. of oxalated plasma plus 7.5 cc. of 0.1 N NaOH are placed in a small dialysis bag of Visking tubing which is suspended in 12.0 cc. of 0.1 N NaOH. Dialysis is allowed to take place for 48 hours. Thereafter the content of drug in the outer NaOH solution is read against a blank obtained by treating similarly plasma from the same source but containing no drug. From these transmission values and the dilution factor the plasma concentration is calculated. Typical recovery data in which known amounts of drug were added to plasma have been summarized in table 1. This method has not been adapted successfully to urine.

The gravimetric recovery of caronamide from urine is a simple procedure which is dependent on its insolubility in acidic aqueous solutions. The total urine volume is recorded and duplicate 100 cc. aliquots of filtered urine are adjusted to approximately pH 3 with glacial acetic acid. The samples are allowed to stand overnight at room temperature. The precipitate is filtered onto tared sintered glass filters, washed with a small amount of dilute

TABLE 1

Demonstrating the determination of caronamide in oxalated plasma by means of a dialysis-spectrophotometric procedure

CARONAMIDE ADDED	PER CENT TRANSMISSION	E	$\frac{C-E}{687}$	DILUTION	CALC.	AV. CALC.
mg./100 cc.					*mg./100 cc.*	*mg./100 cc.*
20	38.0	0.420	0.0061	1:40	24.4	24.4
	38.5	0.414	0.0060		24.0	
	37.5	0.426	0.0062		24.8	
15	54.5	0.264	0.0038	1:40	15.2	15.0
	39.5	0.403	0.0059		23.6*	
	56.1	0.251	0.0037		14.8	
10	66.1	0.180	0.0026	1:40	10.4	11.3
	61.0	0.215	0.0031		12.4	
	64.5	0.190	0.0028		11.2	
5	77.0	0.114	0.0017	1:40	6.8	5.8
	76.8	0.119	0.0017		6.8	
	85.0	0.070	0.0010		4.0	

* Omitted from average.

acetic acid, dried and weighed. The weight of the precipitate is expressed in mg./100 cc., and the melting point of the crystals usually is determined as a check on the identity and extent of contamination.

This method removes caronamide from urine, leaving behind its metabolic products. When high concentrations of caronamide are present a period for precipitation of four to eight hours is sufficient. Crystallization should *not* be allowed to take place in a refrigerator, since under such conditions other substances precipitate also. The method has greatest reliability for the recovery of caronamide from normal urine. Recovery figures for caronamide added to urine are given in table 2.

A colorimetric method has been devised by Ziegler and Sprague (5) for the determination of caronamide in plasma and urine. This method is based on the reductive cleavage of caronamide in the presence of a powdered nickel-aluminum alloy ("Raney catalyst alloy") in alkaline solution to yield p-aminobenzoic acid. The p-aminobenzoic acid is then diazotized and coupled with N(1-naphthyl)-ethylene diamine dihydrochloride. Table 3

gives representative recovery data obtained by the use of this method when caronamide is added to plasma and urine.

It has been our experience that while this method is suitable for caronamide determinations it does not differentiate that agent from certain of its metabolic products. This has been noted in the course of studies on the elimination of caronamide that are in progress in this laboratory, and which will be reported at a later date.

A spectrophotometric method for the estimation of caronamide in plasma and urine has been developed by Brodie and Earle (6). This method is dependent on the extraction of the drug from acidic urine or plasma into chloroform and the reextraction from chloroform into 0.1 N sodium hydroxide. The alkaline solution is read against a suitable blank in a

TABLE 2

The gravimetric recovery of caronamide added to urine

AMOUNT CARONAMIDE ADDED	AMOUNT CARONAMIDE RECOVERED	PERCENTAGE RECOVERY
mg	*mg.*	
250	250.5	100
250	247.7	99
250	237.0	94
500	494.0	99
500	481 5	96

TABLE 3

The recovery of caronamide from plasma and urine by the method of Ziegler and Sprague (5)

CARONAMIDE ADDED	CARONAMIDE RECOVERED	PERCENTAGE RECOVERY
Plasma		
mg /100 cc	*mg /100 cc*	
50.0	46.0	92
40 0	38.0	95
25 0	21 0	84
21 0	21 0	100
12 5	12 0	96
Urine		
100.0	90 0	90
200.0	198 0	99
300.0	300 0	100

Beekman Spectrophotometer at a wavelength of 280 5 mμ. In our hands there is a plasma blank of about 0 3 mg /100 cc. in normal samples. In urine samples diluted 1-20 this blank is negligible. For our instrument an extinction coefficient of 68 3 has been established Figures for the recovery of caronamide from plasma and urine are included in table 4   It is the opinion of Brodie and Earle (6) that this method measures caronamide *per se*   This appears to be true from our experience to date

THE RENAL CLEARANCE OF CARONAMIDE.   In the theoretical considerations of the mode of action and renal elimination of this compound its clearance as-

sumes importance. The enzymologic hypothesis which led to the synthesis
of caronamide has been the competitive inhibition of a definitive component
of the system responsible for the tubular excretion of penicillin. Also, it was
desired that the compound should be essentially refractory to the action of
this definitive mechanism. This is in fundamental contrast with the action
and elimination of PAH which is excreted by the tubules as rapidly as is peni-
cillin.

It seemed most desirable then that the renal elimination of caronamide be
limited to glomerular filtration with little if any reabsorption by the tubules.
It would not seem probable that a compound could inhibit tubular excretion, be

TABLE 4

The recovery of caronamide from plasma and urine as determined by
the method of Brodie and Earle (6)*

CARONAMIDE ADDED	CARONAMIDE RECOVERED	PERCENTAGE RECOVERY
Plasma		
mg./100 cc.	mg./100 cc.	
5.026	4.458	88.7
	4.428	88.0
4.706	4.357	93.0
	4.327	92.0
Urine		
100.00	98.55	98.6
	98.55	98.6
100.00	97.05	97.1†
	97.2	97.2†
3.005	2.985	97.3
	2.940	97.8

* Ten minute extraction periods were used.

† Duplicate samples and blanks precipitated with cadmium sulfate (1:15) before taking
through the procedure described.

reabsorbed actively by the tubules, and yet not inhibit some useful reabsorptive
process.

Early in this research analytical methods were inadequate for the direct
determination of the renal clearance of caronamide. Therefore it was necessary
to use an indirect measure to estimate the over-all elimination of the drug.

A number of recent reports have described the determination of glomerular
filtration rate from the falling plasma concentration of intravenously adminis-
tered mannitol (7). This is based on the hypothesis, supported by experimental
evidence, that its falling plasma concentration should be a function of glomerular
filtration rate, after an initial period during which it is distributed in the body.

Actually, the logarithm of its falling plasma concentration is a linear function of time under these conditions.

It seemed desirable to compare the slope of the falling plasma concentration of caronamide with that for mannitol. Thus if the slope of the caronamide curve were less than that for mannitol its elimination (the summation of its alteration in the body and excretion, actually) would be less than glomerular filtration rate, assuming the volume-distribution of caronamide to be as great as for mannitol.

The experiments were designed as follows: Dogs were given mannitol, creatinine and caronamide intravenously at dosages that would yield drug plasma
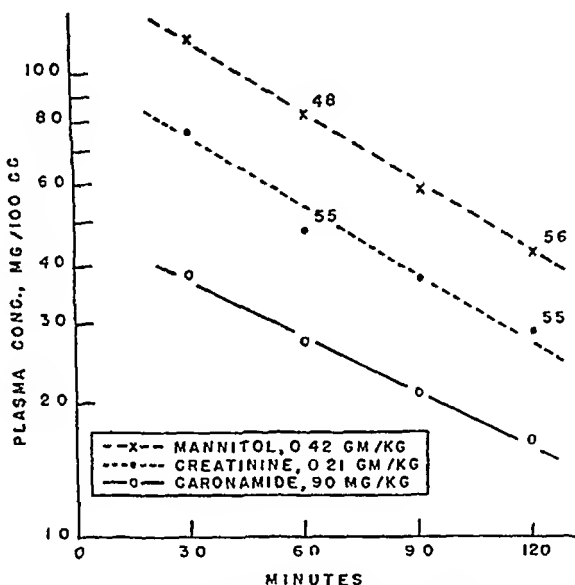
FIG. 1. The falling plasma curves for mannitol, creatinine and caronamide following their intravenous administration as a single injection over a period of 8 minutes. The numerals on the mannitol and creatinine curves indicate the clearance values obtained simultaneously for the two agents, expressed as cc /min

concentrations over a period of time within the range of analytical accuracy or usefulness for the determination of their clearances. At half-hour intervals blood samples were taken for the determination of mannitol, creatinine, and caronamide. At points on the curves indicated by numerals conventional simultaneous 10 minute mannitol and creatinine clearances were performed. These served as a cross check on the experiments and their interpretation.

The protocol and results of such an experiment are given in figure 1. It may be seen that the curves for mannitol and creatinine are essentially parallel and that the clearances indicated thereon are in fair agreement. The curve for caronamide is reproducible and is somewhat flatter than for mannitol.

From these data one may conclude that the over-all elimination of caronamide approaches glomerular filtration rate in the dog. By inference there appears to be no renal tubular excretion of the drug since over a considerable range of plasma concentrations the slope of the curve is uniformly no greater than for mannitol or creatinine whose clearances are equivalent to gomerular filtration rate. The plasma concentrations of caronamide in figure 1 were determined by
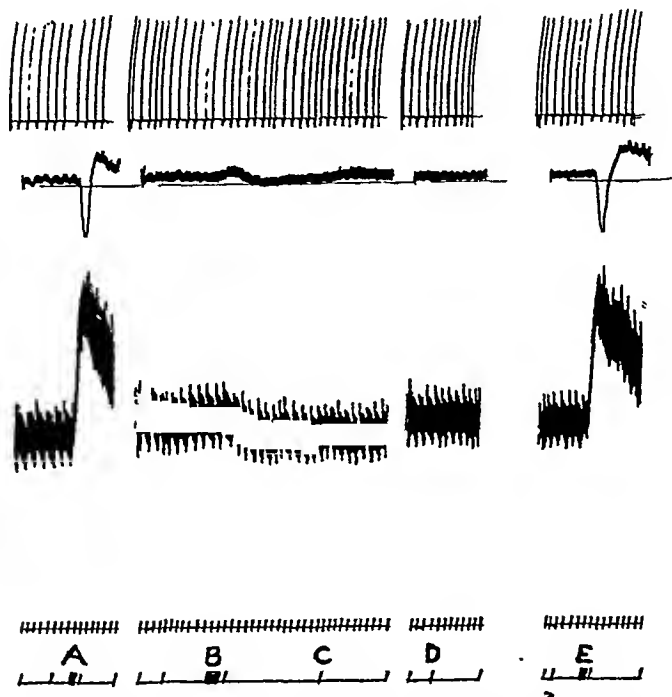
Fig. 2. The effect of epinephrine and caronamide on respiration, renal volume and blood pressure. A, 10γ of epinephrine were administered intravenously over a period of 15 seconds B, a single intravenous dose of 25 mg. of caronamide/kg. was injected, followed immediately by a venoclysis of 30 mg of caronamide/kg./hr. in 5 per cent glucose, infused at a rate of 3 cc./min The short record at D was taken 30 minutes following the initial injection of caronamide. E, a repetition of A wherein 10γ of epinephrine were injected to demonstrate the functional integrity of the preparation. Time, 5 sec. intervals.

the dialysis-spectrophotometric method. However, the results obtained have been confirmed at lower plasma concentrations by the other methods.

Since the method of Brodie and Earle has been available to us it has been possible to determine the clearance of caronamide directly. Table 5 summarizes a simple experiment wherein triplicate caronamide clearances have been performed. The dosage of the drug and the time intervals correspond to those in

experiments wherein the clearance of penicillin (not corrected for plasma binding) was reduced to or below glomerular filtration rate (2). In this experiment the clearance ratio for caronamide was 0.45 to 0.50. The example in table 5 was selected to demonstrate the constancy of caronamide clearance (av. 28 2 cc./min.) determined by the Brodie and Earle method during a changing urine flow. More detailed studies regarding its clearance will be reported later. A personal communication from Dr. Earle reported that the average for caronamide clearance ratios in their studies has been 0.50.

We have not calculated filtration fractions for caronamide, since as yet we have arrived at no reliable figures for its binding on plasma proteins. Brodie and Earle have indicated a considerable binding of the drug. Preliminary evidence obtained by Dr. H. M. Peck in this laboratory indicates that the extraction ratio for renal venous plasma concentrations of caronamide compared to

TABLE 5

The renal clearance of caronamide

Protocol · Dog 183, wt. 16.1 kg.

PERIOD	CARONAMIDE		CREATININE CLEARANCE	CLEARANCE RATIO	URINE FLOW
	Plasma conc	Clearance*			
hr. min.	mg./100 cc.	cc./min.	cc./min.		cc /min
−2:00	600 cc. water, p.o.				
−1:00	400 cc water, p.o				
−0:05	Blood and urine blank obtained				
0:00	Caronamide priming dose 25 mg./kg , i.v. Maintenance dose 30 mg / kg /hr. in 5 per cent glucose infused at 3 cc./min.				
0:35	6 92	29.5	63.3	0.47	0.60
0:45	7.60	27.1	60 9	0.45	1.40
0:55	7.13	28 1	55.5	0.50	2.65

* Not corrected for plasma binding.

femoral arterial concentrations at equilibrium is of the same order but no greater than the simultaneous creatinine ratio in the dog. Indirectly, from the latter data, it might be supposed that the filtration fraction for caronamide approaches 1.0 as a theoretical limit.

THE EFFECT OF CARONAMIDE ON THE RENAL CLEARANCE OF COMPOUNDS OTHER THAN PENICILLIN. In the initial paper of this series it was pointed out that this compound desirably should not alter the function of other transport mechanisms at plasma concentrations sufficient to inhibit maximally the tubular excretion of penicillin (3). We have studied the effects of caronamide at a priming dose of 25 mg./kg. and maintenance dose of 30 mg./kg./hr. on 1) the Tm of glucose reabsorption, 2) the Tm of arginine reabsorption, 3) sulfadiazine and sulfathiazole clearances, 4) urea clearance, 5) creatinine clearance as an index of glomerular filtration, 6) the Tm of PAH tubular excretion, 7) PAH clearance at very low plasma concentrations, and 8) phenolsulphonphthalein clearances.

At this dosage of caronamide the renal tubular excretion of penicillin was suppressed to the point that its clearance ratio was less than 1.0 (2).

These clearances were performed in much the same manner as has been described for the initial evaluation of the effect of a compound on penicillin clearance. To avoid a repetitive description of methods the protocol for each type of experiment is included in a table giving typical data for each study. In general there were successive duplicate normal clearances on each agent followed by duplicate compound clearances after the caronamide blood level had been given time to approximate equilibrium.

1) *Effect on Glucose Tm.* We were particularly interested in the effect of caronamide on the maximal reabsorptive capacity for glucose. It was found in the pathologic (8) and clinical (9) evaluations of this drug that when very high dosages were administered a non-fermentable reducing substance that was not

TABLE 6

The lack of effect of caronamide on the Tm of glucose

PERIOD	GLUCOSE		CREATININE CLEARANCE	URINE FLOW
	Plasma conc.	Tm		
hr.:min.	*mg./100 cc.*	*mg./min.*	*cc./min.*	*cc./min.*
−1:00	600 cc. H_2O, p.o.			
0:00	3 gms. creatinine plus 25 gms. glucose in 250 cc. H_2O, p.o.			
0:20	Began infusion of 20% glucose plus 0.5% creatinine at 6 cc./min.			
0:55	584	203	58.9	9.18
1:05	680	234	59.3	8.89
1:15	Caronamide priming dose, 25 mg./kg. i.v., maintenance dose 30 mg./kg./hr. in soln. containing 20% glucose plus 0.5% creatinine.			
1:45	740	246	63.6	7.05
1:55	740	208	56.3	5.80

glucose sometimes appeared in the urine. It seemed desirable to know whether the Tm for glucose had been lowered by caronamide, even though it was not decreased sufficiently to yield frank glycosuria at normal blood sugar levels.

The protocol given with the results of a representative experiment in table 6 should suffice to give the design of this work. It may be seen that at a dosage that will abolish the tubular excretion of penicillin caronamide does not influence the maximal rate (Tm) at which glucose is reabsorbed by the renal tubules.

2) *Effect on Arginine Tm.* Although it is difficult to say that the renal elimination of one essential group of nutritional factors is more important than another, certainly the potential effect of this compound on the reabsorption of amino acids should receive considerable attention. Recently we have studied the renal clearances of amino acids with the aid of microbiologic assay technics that permit the determination of naturally occurring optical forms of individual

amino acids. Of those that have been studied thus far by these methods (10, 11) only lysine and arginine have been found to have determinable values for Tm at plasma concentrations tolerated by the dogs. Since the natural form of arginine is available in large quantities, and since its Tm is very clearly defined, it was selected for these studies. The methods used for the determination of arginine Tm are described elsewhere (11).

The protocols of these experiments are included together with representative results in table 7. Here again duplicate clearances were run in both the initial

TABLE 7

A. The lack of effect of caronamide on the Tm of arginine
B. Control determination of the Tm of argine

PERIOD	ARGININE			CREATININE CLEARANCE	URINE FLOW
	Plasma conc.	Clearance	Tm		
		Protocol A: Dog 370, wt. 12.8 kg.			
hr min.	mg /100 cc	cc./min.	mg./min.	cc./min.	cc./min.
0:00	2 8 gm. creatinine s.c.				
	Began arginine infusion, 5.0 mg./kg./min. at 3 cc./min., i.v.				
0:05	Arginine priming dose, 3.0 mg./kg., i.v.				
0:25	28.0	11.8	9.1	44.6	4.5
0:35	31.2	13.1	9.6	44.0	3.7
0:40	Caronamide: 25 mg /kg. priming dose, i.v. Maintenance dose 30 mg./kg. /hr. in soln. containing arginine.				
1:15	30 9	28 8	9 9	61.1	5.8
1:25	34.7	23.8	9.0	49.8	5.4
	Protocol B: Dog 370, wt. 12.4 kg.—control experiment				
0:00	Began arginine infusion, 5.0 mg./kg./min. at 3 cc./min. i.v.				
0:05	Arginine priming dose, 3.0 mg./kg., i.v., 2.5 gm. creatinine, s.c.				
0:25	22.8	8.6	9.3	55.4	4.9
0.35	19.8	14.7	9.7	57.4	4.9
1:15	24.7	31.5	9.1	64.6	6.1
1:25	26.9	28.3	9.1	62.0	5.5

and second phase of the tests. It may be seen in the data on the first trial tabulated that the drug did not influence the normal Tm of arginine. However, in the drug phase of the experiment, the renal clearance for arginine increased, as it did for creatinine. This observation appears to be attributable to the amino acid, arginine. This interpretation is borne out in a second experiment that was done several days later on this same dog, table 7. In this latter trial the design was the same as in the previous one in every respect except that caronamide was not administered, the second phase being essentially a control. It may be seen

that the outcome of this experiment was very similar to the former one wherein the drug was administered at the onset of the second phase.   There was no real change in arginine Tm but there was an increase in both arginine and creatinine clearances.   One would expect an increase in arginine clearance with creatinine clearance if the Tm of arginine reabsorption had been reached and the plasma concentration of arginine was constant or rising.   It has been our limited experience that arginine Tm determinations are reasonably reproducible from day to day in normal dogs, as may be seen in these data.

3) *Effect on sulfonamide clearances.* •This group of compounds was selected for study since their clearances represent the filtration and reabsorption of medicinal agents as distinguished from endogenous products.

TABLE 8

The lack of effect of caronamide on the renal clearance of sulfadiazine

Protocol: Dog 370, wt. 12.2 kg.

PERIOD	SULFADIAZINE		CREATININE CLEARANCE	URINE FLOW
	Plasma conc.	Clearance*		
hr.:min.	mg./100 cc.	cc./min.	cc./min.	cc./min.
0:00	Sulfadiazine, 1.0 gm., i.v.			
0:15	Began infusion of 5 per cent glucose, 3 cc./min., i.v.			
0:18	2.5 gm. creatinine, s.c.			
0:40	14.5	14.3	60.6	3.25
0:50	13.6	12.3	64.0	2.80
1:00	Caronamide priming dose 25 mg./kg., i.v. Maintenance infusion 30 mg./kg./hr. in 5 per cent glucose, 3 cc./min., i.v.			
1:35	11.4	16.2	62.6	3.70
1:45	11.4	14.6	59.4	3.40

* Not corrected for plasma binding.

The protocol and results for sulfadiazine given in table 8 may be considered as representative for other sulfapyrimidines and sulfathiazole also.   The renal clearance of these compounds before and concomitant with the administration of caronamide was essentially the same.   Actually one might have predicted some increase in the clearance of sulfadiazine in the drug phase of the experiments since alterations in electrolyte output are sufficient to increase sulfonamide clearances (12).   Evidently the excretion of caronamide did not cause, either directly or through any influence on tubular function, any detectable alteration of the electrolyte content of the urine.

4) *Effect on urea clearance.* These experiments may be taken to represent the effect of caronamide on the clearance of a product of protein metabolism.   The experiments are essentially the same as the preceding ones.   Dosages and other details are given in table 9 together with the results of a typical experiment.   It may be concluded that caronamide does not influence urea clearance.

5) *Effect on glomerular filtration.* One may refer to the preceding renal function studies for evidence that caronamide does not alter glomerular filtration rate, i.e., creatinine clearance. It has been determined that such is the case for man (9a) as well as for dogs.

TABLE 9

The lack of effect of caronamide on the renal clearance of urea

Protocol Dog 84, wt 16 0 kg

| PERIOD | UREA | | CREATININE CLEARANCE | URINE FLOW |
	Plasma conc	Clearance		
hr min	mg /100 cc	cc /min	cc /min	cc /min
0:00	Began urea infusion, 0 5 per cent soln in saline at 3 cc./min , i v.			
0:05	Urea priming dose 20 mg /kg , i v , 3 0 gm. creatinine, s c.			
0:35	21 4	65 1	98 3	3 3
0:47	23 8	53 8	84 3	4.2
0:55	Caronamide priming dose 25 mg /kg , i v. Maintenance dose 30 mg /kg /hr in urea-saline soln at 3 cc /min , i.v			
1:36	26 8	61 8	90 3	8 2
1:45	26 9	59 6	88 5	7 9

TABLE 10

The effect of caronamide on the Tm of PAH (p-aminohippurate)

Protocol Dog 84, wt 15 7 kg

| PERIOD | PAH (P AMINOHIPPURATE) | | | CREATININE CLEARANCE | URINE FLOW |
	Plasma conc	Clearance	Tm		
hr min	mg /100 cc	cc /min	mg /min	cc /min	cc /min.
0:00	Began infusion of 140 mg PAH/kg /hr in 5 percent mannitol soln at 3 cc /min , i v				
0:02	PAH priming dose, 140 mg /kg plus 5 gm. mannitol, i v				
0:03	2 8 gm creatinine, s c				
0:35	29 6	99 0	17 8	68 7	9 3
0:45	28 0	94 6	15 5	67 2	8 2
0:55	Caronamide priming dose 25 mg /kg , i v. Maintenance infusion 30 mg /kg /hr in 5 per cent mannitol soln				
1:30	30 0	71 7	6 4	68 3	8 5
1:40	30 0	71 7	7 1	66 4	8 3

6) *Effect on the Tm of PAH.* It has been pointed out that penicillin and PAH (p-aminohippurate) are excreted by the same (functional) tubular transport mechanism (4). If this be true then caronamide should decrease the Tm of PAH.

From the type of experiment exemplified in the data contained in table 10

it may be concluded that caronamide very materially decreased the maximal rate (Tm) for the tubular excretion of PAH. Here the relative concentrations of PAH and caronamide presented to the excretory tubular transport system were at least 3:1 in favor of the PAH substrate. In one such experiment, wherein the plasma concentration of PAH was about 15 mg./100 cc. and the control Tm was 10.5 mg./min., the coadministration of this same dosage of caronamide reduced the PAH excretion to where the PAH glomerular filtration (calculated) was equal to its UV value, or the total amount excreted per minute.

7) *Effect on the maximal clearance rate of PAH.* At low plasma concentrations PAH clearance has been reported to be equivalent to minimal renal plasma flow (13). However, in the presence of caronamide this relationship breaks down

TABLE 11

The effect of caronamide on the maximal renal clearance of PAH (p-aminohippurate)

Protocol Dog 365, wt 12 5 kg.

PERIOD	PAH		CREATININE CLEARANCE	FILTRATION FRACTION	URINE FLOW
	Plasma conc	Clearance*			
hr min	*mg /100 cc*	*cc /min*	*cc /min*		*cc /min*
0:00	700 cc. H₂O, p o.				
1:00	PAH, 200 mg /kg , p o				
1:40	3 0 gm creatine, s c.				
2:00	0 6	188 0	56 1	0.30	4 7
2:10	0 8	136 8	56 2	0 41	4 4
2:20	Caronamide priming dose 25 mg /kg., i v. Maintenance infusion 30 mg /kg /min. in 5 per cent glucose soln at 3 0 cc /min.				
2:55	0 9	76 2	61 7	0 81	3 4
3:05	0 9	82 2	52 5	0 64	2 6

* Not corrected for plasma binding.

(table 11) since the latter compound inhibits the transport mechanism for the excretion of penicillin and PAH by the tubules.

These results and interpretations are being checked at the present time from the standpoint of simultaneous renal blood flow measurements and extraction ratios for PAH.

8) *Effect on the clearance of phenolsulfonephthalein (PSP).* This compound was shown conclusively by Marshall and his associates to be excreted by the tubules (14). This being the case, it was of interest to determine the effect of caronamide on the clearance of PSP. If the tubular excretory mechanism for penicillin, PAH and PSP were at least functionally the same, caronamide should inhibit the excretion of PSP as well as the other compounds. A typical protocol and the results of such an experiment are presented in table 12.

It is evident from the data in table 12 that caronamide decreased the clearance of PSP from a control clearance ratio of 1.63 (av.) to 0 41 (av.), or to 41 per cent

5) *Effect on glomerular filtration.* One may refer to the preceding renal function studies for evidence that caronamide does not alter glomerular filtration rate, i e , creatinine clearance It has been determined that such is the case for man (9a) as well as for dogs

TABLE 9

The lack of effect of caronamide on the renal clearance of urea

Protocol Dog 84, wt 16 0 kg

PERIOD	UREA		CREATININE CLEARANCE	URINE FLOW
	Plasma conc	Clearance		
hr min	mg /100 cc	cc /min	cc /min	cc /min
0:00	Began urea infusion, 0 5 per cent soln in saline at 3 cc /min , i v			
0.05	Urea priming dose 20 mg /kg , i v , 3 0 gm creatinine, s c			
0:35	21 4	65 1	98 3	3 3
0:47	23 8	53 8	84 3	4 2
0:55	Caronamide priming dose 25 mg /kg , i v Maintenance dose 30 mg /kg /hr in urea saline soln at 3 cc /min , i v			
1:36	26 8	61 8	90 3	8 2
1:45	26 9	59 6	88 5	7 9

TABLE 10

The effect of caronamide on the Tm of PAH (p aminohippurate)

Protocol Dog 84, wt 15 7 kg

PERIOD	PAH (P AMINOHIPPURATE)			CREATININE CLEARANCE	URINE FLOW
	Plasma conc	Clearance	Tm		
hr min	mg /100 cc	cc /min	mg /min	cc /min	cc /min
0:00	Began infusion of 140 mg PAH/kg /hr in 5 percent mannitol soln at 3 cc /min , i v				
0:02	PAH priming dose, 140 mg /kg plus 5 gm mannitol, i v				
0:03	2 8 gm creatinine, s c				
0:35	29 6	99 0	17 8	68 7	9 3
0:45	28 0	94 6	15 5	67 2	8 2
0:55	Caronamide priming dose 25 mg /kg , i v Maintenance infusion 30 mg /kg /hr in 5 per cent mannitol soln				
1:30	30 0	71 7	6 4	68 3	8 5
1:40	30 0	71 7	7 1	66 4	8 3

6) *Effect on the Tm of PAH.* It has been pointed out that penicillin and PAH (p-aminohippurate) are excreted by the same (functional) tubular transport mechanism (4). If this be true then caronamide should decrease the Tm of PAH.

From the type of experiment exemplified in the data contained in table 10

throughout the experiment. In the initial phase of the experiment intravenous injections of small amounts of epinephrine were made to judge the adequacy of the instrumentation.

After it was ascertained that the set up would respond to variations in functions, a single intravenous "priming" injection of 25 or 30 mg. of caronamide/kg. as a 10 per cent solution was made over a duration of about 15 seconds. This

TABLE 13

The effect of epinephrine and caronamide on the systemic blood pressure, heart rate, respiratory rate and kidney volume of dogs

After a 30 minute infusion of glucose a single intravenous dosage of 25 mg./kg. of caronamide was followed by the venoclysis of 30 mg. caronamide/kg./hr. in 5 per cent glucose.

DOG WGT.	PROTOCOL: I.V. ADMINISTRATION	BLOOD PRESSURE RISE (+) OR FALL (−) MM.	HEART RATE*	RESP. RATE*	KIDNEY VOLUME†			
					1st change		2nd change	
					± mm.	Duration	± mm.	Duration
11.7	50 γ epinephrine initial caronamide; after 30 min.	+100 −8 +4	148/230 140/152 148	6/4 6/8 9	−21 +2 +2	20″ 10″	+8 −1	1′ 3′
11.4	20 γ epinephrine initial caronamide; after 30 min.	+44 −6 −2	146/176 136/154 144	12/9 14/15 18	−10 0 0	5″		
9.6	10 γ epinephrine initial caronamide; after 30 min.	+16 −8 −10	144/166 144/144 156	14/12 14/14 20	−5 −2 0	20″ 160″	+1	3′
9.4	10 γ epinephrine initial caronamide; after 30 min. 10 epinephrine	+60 −5 0 +54	116/132 112/116 116 116/128	6/4 6/5 5 6/4	−14 +1 −½ −13	20″ 30″ 20″	+5 −1 +5	60″

* 146/166 and 8/7 means the control heart and respiratory rates were 146/min. and 8/min. respectively and the heart and respiratory rates after administration of the drug were 166/min. and 7/min. respectively.

† ± mm. refers to the change as measured on the kymograph record. A (+) change indicates a decrease in kidney volume and a (−) change an increase in kidney volume.

was followed immediately by the venoclysis of caronamide at a dosage of 30 mg./kg./hr. in 5 per cent glucose at a rate of 3.9 cc./min.

Continuous kymographic records of kidney volume, arterial blood pressure and respiratory rate and volume were taken during the epinephrine and caronamide injection and again 30 minutes following the continuous infusion of caronamide.

Epinephrine in dosages of 10 or 20 micrograms consistently increased blood pressure and heart rate but decreased respiratory rate and kidney volume. The

of glomerular filtration rate. This is not surprising for if tubular excretion of PSP were totally suppressed its clearance would be limited by its ultrafiltration at the glomerulus, assuming no active or passive reabsorption. Grollman found that the amount of PSP in an ultrafilterable form in dog plasma was 25 per cent of the total concentration in this range and at an albumin content of 4.8 per cent (15). It is unlikely that the albumin content of the plasma of this dog was as high as 4.8, due to hemodilution secondary to the intake of a liter of water at the start of the experiment. However, in other experiments the clearance ratio of PSP following this same dose of caronamide has been less than in this example, table 12.

The effect on renal volume, systemic blood pressure and respiration. It seemed quite certain that the decrease in the maximal clearance of PAH did not represent

TABLE 12

The effect of caronamide on the renal clearance of phenolsulfonephthalein

Protocol: Dog 225, wt. 22.8 kg.

| PERIOD | PHENOLSULFONEPHTHALEIN | | CREATININE CLEARANCE | CLEARANCE RATIO | URINE FLOW |
	Plasma conc	Clearance*			
hr min .	mg /100 cc.	cc /min.	cc /min.		cc /min
−0:30	Began i v. infusion of 5 per cent glucose solution at 3 cc./min., i.v.				
0:00	90 mg. sodium phenolsulfonephthalein plus 3 gm. creatinine, s.c.				
0:35	0 45	143 9	94.3	1.53	6.5
0:47	0.35	164.7	95.4	1 73	6.2
0:52	Caronamide priming dose 25 mg./kg , i.v. Maintenance dose 30 mg./kg /hr. in 5 per cent glucose infused at 3 cc./min., i.v.				
1:25	0.33	33 2	89 1	0.37	5.8
1:35	0.31	35.4	81.0	0.44	5.8

* Not corrected for plasma binding.

a decrease in renal blood flow but rather an inhibition of the tubular excretion of the compound. Still, it was desirable to study the effect of caronamide on the general and local changes in the systemic vascular bed.

For this purpose dogs were anesthetized with vinbarbital sodium, 45 mg./kg. intravenously. The left carotid artery was cannulated and connected to a mercury manometer. The trachea was cannulated and respiration was recorded by the Gaddum method. Both vagi were doubly ligated in the neck and sectioned between the ties. An oncometer was placed on the left kidney to record changes in renal volume. The right femoral vein was exposed for injections. Heart rate was obtained by auscultation.

Immediately following surgery the dog was given 40 cc. of tap water/kg. by stomach tube. Thirty minutes thereafter an intravenous infusion of 5 per cent glucose at 3.9 cc./min. was begun. The infusion at this rate was continued

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initial effect on kidney volume was striking, though transient, and was followed occasionally by a secondary increase in kidney volume.

The initial 25 mg./kg. dose of caronamide produced a fall in blood pressure of 2 to 5 mm. of mercury; the heart rate either did not change or increased to a maximal change of 18 beats per minute.   There was no real change in respiratory rate.   The kidney volume either did not change in some experiments or increased to a barely detectable extent for a duration of 10 to 160 seconds in other instances.   There was no effect of the continued infusion of caronamide during these experiments   In our opinion there were no effects of caronamide on kidney volume, blood pressure, respiratory and heart rate, aside from the hypertonicity of the priming dose and the volume of fluid administered during the venoclysis. A kymographic record of such an experiment is illustrated in figure 2.   Table 13 summarizes the data on the last 4 of 7 such experiments.   These data were obtained by Dr. H. M. Peck of this department.

<div style="text-align:center">SUMMARY</div>

The chemical properties of caronamide and methods for its determination in plasma and urine have been summarized

Data have been presented from which the following four conclusions may be derived:

1. The renal clearances and over-all rate of elimination of caronamide appear tentatively to be equivalent to glomerular filtration rate in the dog.

2. The inhibitory effect of caronamide is on a renal tubular excretory transport mechanism; hence other compounds, i e. p-aminohippurate and phenolsulfonephthalein, in addition to penicillin that are excreted by this system are affected similarly.

3. Caronamide did not influence other renal functions as indicated by the Tm of glucose or arginine, urea clearance, sulfonamide clearance, or creatinine clearance (glomerular filtration rate).

4. At concentrations of the drug that suppressed completely the tubular excretion of penicillin (2) it did not influence significantly heart or respiratory rate, systemic blood pressure, or kidney volume

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feine was used the final concentrations varied from 0.4 mgm. to 2.0 mgm. present in the 1.8 cc. of Ringer's solution in the Warburg vessel. The results are reported in terms of mm.³ of oxygen used per mgm. wet weight of tissue per hour.

TABLE 1

Effect of theobromine derivatives on oxygen consumption of rat's diaphragm*

THEOBROMINE DERIVATIVE	CONC.†	OXYGEN CONSUMPTION		PER CENT CHANGE	NUMBER OF EXP.
		Without	With		
	mgm.	mm.³ O_2 per hour	mm.³ O_2 per hour		
Methyl (caffeine)......................		1.18	1.52	29	14
Ethyl................................	1.0	1.44	1.85	28	3
n-Propyl.............................	1.0	1.28	1.63	27	3
n-Butyl..............................	1.0	1.01	1.35	33	3
Allyl................................	1.0	1.26	1.78	41	3
Methoxyethyl.........................	1.0	0.90	1.61	79	3
Crotyl...............................	1.0	1.19	1.70	43	3
Isoamyl..............................	1.0	1.34	1.48	10	3
Methallyl............................	1.0	1.18	1.45	23	3
Theobromine.........................	1.0	1.28	1.54	20	3
Theophylline.........................	1.0	1.23	1.72	40	2

* Oxygen consumptions are the average of the results of the number of experiments given in the last column.

† Total amount present in 1.8 cc. Ringer's glucose solution.

TABLE 2

Effect of caffeine on oxygen consumption of rat's diaphragm

AMOUNT	OXYGEN CONSUMPTION		PER CENT CHANGE	NUMBER OF EXP.
	Without	With		
mgm.	mm.³ O_2 per mgm. per hour	mm.³ O_2 per mgm. per hour		
2.0	1.10	1.31	19	3
2.0	1.28	1.57	23	3
1.0	1.23	1.55	26	2
0.4	1.59	1.63	2	3
0.6	1.23	1.73	41	3
1.0	1.11	1.44	30	3
2.0*	1.07	1.24	16	3
2.0	1.19	0.98	−18	3
2.0†	1.24	0.90	−27	3

* No glucose.

† Caffeine placed in Ringer's solution before experiment.

RESULTS. The results are given in tables 1 and 2 and in figure 1. It may be seen in table 1 that all of the compounds tested stimulated the oxygen consumption of the isolated rat's diaphragm. There was a difference in the increase of oxygen consumption with the various compounds, for example, the average for

THE EFFECTS OF CAFFEINE AND THEOBROMINE DERIVATIVES ON OXYGEN CONSUMPTION OF MUSCLE[1,2]

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There has been considerable work on the effect of caffeine on the oxygen consumption of isolated frog muscle. Meyerhof (1) states that 0.09 per cent caffeine increased the respiration of resting frog muscle 85 per cent. Fenn (2) found that 0.06 per cent caffeine caused a permanent elevation in oxygen consumption while higher concentrations produced a large initial increase which was followed by a decrease to zero. Saslow (3) continued this work on frog muscle by measuring respiratory quotients and metabolic activity. He found that caffeine increased the oxygen consumption from four to twenty-four times and the respiratory quotient of the muscle was one under the influence of this drug. In contrast to these numerous observations of the effect of caffeine on the oxygen consumption of isolated frog muscle is the lack of experiments on warm blooded muscles. Also, no experiments have been made with various derivatives of theobromine on isolated muscle. Scott and Chen (4) and Scott, Anderson and Chen (5) have studied recently the effects of caffeine and a series of 1-substituted theobromine derivatives on stimulation of motor activity, respiration and diuresis. Since all of these compounds were active in the intact animal, it was decided to study the effect of these compounds on the oxygen consumption of the isolated diaphragm of the rat.[3] All of the derivatives studied had the substitutions in the 1 position

METHODS. The diaphragm of the rat was dissected out and prepared for the manometric vessels by the method described by Gemmill (6). About 100 mgm. of muscle was placed in 1.6 cc. of Ringer's solution containing glucose while 0 2 cc of caffeine or the theobromine derivative in 0 9 per cent NaCl solution was placed in the side cup of the Warburg vessel. Several of the solutions of the theobromine derivatives had to be heated to get the compounds into solution. 0 2 cc of 20 per cent potassium hydroxide and a small piece of filter paper were placed in the center well to absorb the carbon dioxide formed during the experiment. Oxygen was passed through each vessel for six minutes while it was being shaken in the water bath at 37.5° C. After this period, the side tubes were closed and the vessels shaken for an additional five minutes before the first readings were made. Then the stopcocks were closed and the caffeine or theobromine derivative was spilled over into the reaction vessel. With each caffeine or theobromine derivative experiment a control experiment was made in which 0 2 cc of 0 9 per cent sodium chloride solution was placed in the side tube. Manometer readings were made every fifteen minutes for one hour When caf-

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tion.   In this series of experiments on isolated muscle, all of the compounds gave an increased oxygen consumption with crotyl theobromine giving the greatest change and isoamyl theobromine the least.

DISCUSSION OF RESULTS.   Since all the compounds in this series gave a respiratory stimulation, these experiments throw no light on the mechanisms involved in this effect.   It is of interest, however, to compare the effects with those on frog muscle.   Respiration of frog muscle is much more sensitive to caffeine than the rat's diaphragm, for at no time were oxygen consumptions noted in this series comparable to those obtained on frog muscle.

It may be that all of these compounds produce contracture which would increase the oxygen consumption slightly.   For this reason, a study is being made of the effect of caffeine on glycolysis in order to obtain a better knowledge of the mechanisms involved in caffeine stimulation.

CONCLUSIONS

1. A series of 1-substituted theobromine compounds was used in a study of their effects on oxygen consumption of the isolated diaphragm of the rat.

2. All of the compounds studied increased this activity.

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the three experiments with isoamyl theobromine was 10 per cent while crotyl theobromine gave 43 per cent. Due to the variability in the individual results, no significance can be attached to these quantitative differences.

The results of the experiments with caffeine are given in table 2 and the time course of the oxygen consumption of a typical experiment is presented in figure 1. From table 2, it may be seen that there is a limiting concentration for this stimulation for no effect was obtained with 0.4 mgm. caffeine present in the solution while 0.6 mgm. or greater concentrations gave a decided result. If glucose was absent from the medium, the degree of stimulation was decreased. If caffeine was added to the Ringer's solution before the experiment and not placed in the side tube, there was no stimulation of oxygen consumption, but an inhibition. The primary effect of caffeine on oxygen consumption may be seen in figure 1. The
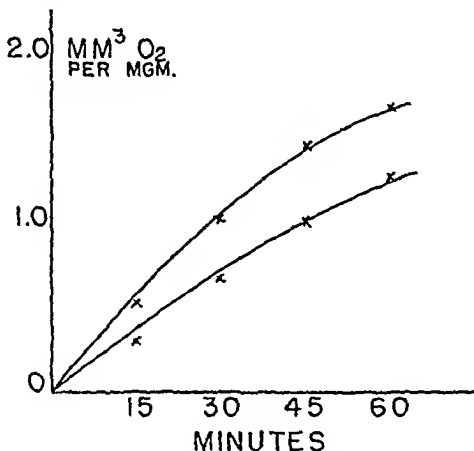
FIG. 1. EFFECT OF CAFFEINE ON OXYGEN CONSUMPTION OF RAT'S DIAPHRAGM

0.1 per cent glucose in Ringer's solution. Upper curve, Ringer's solution with 2 mgm. caffeine; lower curve, Ringer's solution without caffeine. Temperature, 37.7°C. Results given in terms of mm.³ oxygen per mg of wet weight tissue

greatest stimulation occurs in the first fifteen minutes, after which the velocity of the uptake of oxygen in the control and the caffeinized muscle is practically the same.

The magnitude of the oxygen consumption for forty-one control experiments was 1.23 mm.³ of oxygen per mgm. of muscle. This figure is slightly higher than the value of 0.97 reported before by Gemmill (7) on the similar preparation with 0.2 per cent glucose in the medium.

It is also of interest to compare these results obtained on the oxygen consumption of isolated rat muscle with the change in respiratory activity obtained by Scott, Anderson and Chen (5) in trained unanesthetized dogs. In their series, they found that all of these compounds increased the respiratory activity of their animals. Propyl theobromine was most active in causing respiratory stimula-

tate and fluoride in the glycolytic reaction. The concentrations of these substances are given in connection with the tables presenting the results. Phosphate determinations were made by the method of Fiske and Subharow (7) using a photoelectric colorimeter for the final readings. In these experiments additional phosphate was added in the form of 0.1 M phosphate buffer adjusted to a pH of 7.4.

RESULTS. The results are given in tables 1 to 6 and in fig. 1. A typical response to caffeine is shown in fig. 1. It may be seen in this figure that when caffeine is present there is a primary stimulation followed by an inhibition. In this experiment the curves crossed after fifty-three minutes. From the slope of the two curves, it is apparent that the stimulation is only in the very early part of the experiment.
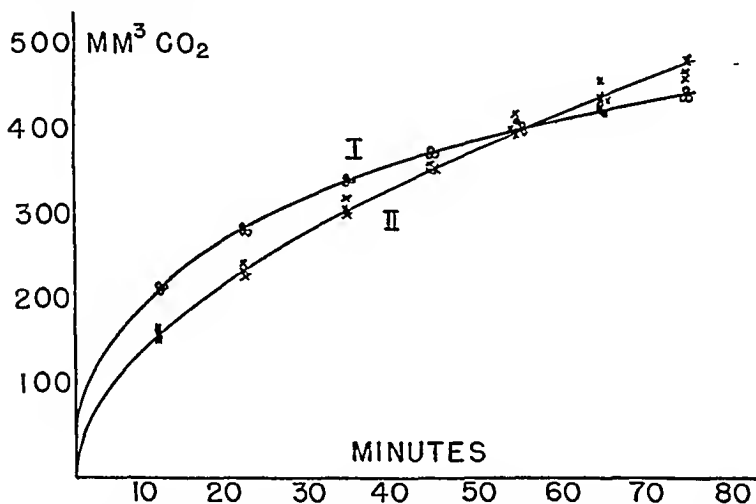
FIG. 1. THE EFFECT OF CAFFEINE ON GLYCOLYSIS

Curve I, with caffeine; Curve II, without caffeine. Concentrations of solutions: 1.2 cc. of muscle extract; 0.4 cc. of 1.3 per cent sodium bicarbonate; 0.2 cc. of 4.0 per cent glycogen; 0.4 cc. of 0.3 per cent caffeine; 95 per cent nitrogen and 5 per cent carbon dioxide; 25.0°C.

The same phenomenon was observed to a greater or lesser degree with the various theobromine derivatives. A table (table 1) is given for the per cent differences between the two rates of glycolysis at the end of ten minutes and at the end of one hour for each derivative studied. The majority of the theobromine derivatives gave also a primary stimulation followed by a secondary inhibition. The exceptions to this finding were butyl theobromine which gave a slight inhibition and methallyl theobromine which gave very little change either at the end of the ten or sixty minute periods. In fact, in two of the early caffeine experiments, very little difference was noticed at the end of the ten minute period (+8 and +6 per cent). Theophylline, urethane and 2-aminopyrimidine have no effect on the velocity of glycolysis. These substances served as controls for the previous experiments.

THE EFFECTS OF CAFFEINE AND THEOBROMINE DERIVATIVES ON MUSCLE GLYCOLYSIS[1,2]

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In a former paper (Gemmill, 1) the effect of caffeine and theobromine derivatives on oxygen consumption of the isolated rat's diaphragm was described. It was found that caffeine and numerous theobromine derivatives with substitutions in the 1 position stimulated the respiratory metabolism of the isolated muscle preparation. It was impossible to tell from these experiments whether or not the stimulation was a non-specific one resulting from contracture of the muscle or whether it was specific from stimulation of some definite part of the metabolic process occurring in muscle. Therefore it was decided to extend this study by testing the effects of the same group of substances on glycolysis and to make an intensive study of caffeine on this mechanism.

There have been a few reports on the effect of caffeine on glycolysis and these reports concern only work on intact muscle. Meyerhof (2) states that the addition of caffeine increases the lactate formation of muscle. This work was continued and reported in detail by Matsuoka (3). David (4) also has shown a large change in lactate in caffeine contracture. Hill (5) demonstrated that after application of caffeine to muscle there was a prolonged spontaneous liberation of heat lasting until the muscle became inexcitable. This heat liberation occurred under both anerobic and aerobic conditions.

METHODS. The methods used were similar to those employed by Gemmill and Hellerman (6) in their study of reversible inactivation of glycolysis. The muscles of the hind legs of frogs were quickly dissected and placed in an ice cold mortar. For every part of muscle, 1.5 parts of cold water were added and the mixture ground with sand. After centrifuging, the supernatant fluid was used for the determinations. Generally 1.0 cc. of extract was used in each Warburg vessel with 0.4 cc. of 1.3 per cent sodium bicarbonate and 0.4 cc. of 0.3 per cent caffeine or theobromine derivative.[3] Then 0.2 cc. of 4.0 per cent glycogen was placed in the side tube. After the vessels were put into a water bath (25.0° C), 95 per cent nitrogen and 5 per cent carbon dioxide was passed through the vessels for six to eight minutes. Following this period, the side tubes were closed and the vessels shaken for an additional five minutes before the first readings were taken, the stop-cocks closed and the glycogen spilt over into the reaction vessels.

After caffeine and the various theobromine derivatives were studied with this method, additional experiments were made using (1) phosphate (2) magnesium sulfate (3) iodoace-

reaction vessels in addition to caffeine. In some of these experiments phosphate determinations were made in addition to the manometric readings. When caffeine was added to a solution with magnesium sulfate but without additional phosphate, only inhibition of glycolysis was observed (table 2); however, when caffeine was added to the extract with additional phosphate, stimulation was found (table 3). The addition of magnesium sulfate in the presence of phosphate

TABLE 3

Effects of caffeine and phosphate on glycolysis

EXP.	GLYCOLYSIS (MM.³ CO₂)					
	10 minutes			60 minutes		
	Phosphate without caffeine	Phosphate with caffeine	Diff.	Phosphate without caffeine	Phosphate with caffeine	Diff.
			per cent			*per cent*
1	122	175	43	360	394	9
2	84	103	23	307	295	−4

Each experiment is the average of two determinations. Each vessel contained 1.0 cc. extract (1 part muscle, 1.5 parts water); 0.2 cc. of 0.1 M phosphate; 0.2 cc. of 4.0 per cent glycogen; 0.4 cc. of 1.3 per cent sodium bicarbonate. Caffeine concentration, 0.4 cc. of 0.3 per cent. Final volume of solution in each vessel adjusted to 2.2 cc. with water. 95 per cent nitrogen and 5 per cent carbon dioxide. Temperature, 25.7° C.

TABLE 4

Effects of caffeine, magnesium sulfate and phosphate on glycolysis

EXP.	GLYCOLYSIS (MM.³ CO₂)					
	10 minutes			60 minutes		
	Caffeine + phosphate		Diff.	Caffeine + phosphate		Diff.
	Without MgSO₄	With MgSO₄		Without MgSO₄	With MgSO₄	
			%			%
1*	179	261	45	292	475	63
2	149	184	23	339	475	40
3*	156	279	79	316	572	81

* Experiments 1 and 3 are given as the average of two determinations.

Concentrations of solutions: 1.0 cc. of extract (1 part muscle, 1.5 parts water); 0.2 cc. of 2.6 per cent sodium bicarbonate; 0.2 cc. of 0.1 M phosphate; 0.2 cc. of 4.0 per cent glycogen; 0.2 cc. of 1.0 per cent magnesium sulfate and 0.4 cc. of 0.3 per cent caffeine. Final volume adjusted to 2.2 cc. in each vessel with water. 95 per cent nitrogen and 5 per cent carbon dioxide. 25.7°C.

and caffeine also produced a stimulation (table 4). Therefore, the results (table 1) observed in the earlier experiments in which primary stimulation was followed by secondary inhibition are probably related to the concentration of phosphate or magnesium ions. If large amounts of free phosphate are present, stimulation is obtained. All of these reactions are inhibited when sodium fluoride and iodo-acetic acid are present (table 5).

TABLE 1

*Effect of caffeine and theobromine derivatives on glycolysis**

THEOBROMINE DERIVATIVE	CONC.†	GLYCOLYSIS						NO. OF EXP.
		10 minutes			60 minutes			
		without mm.³ CO₂	with mm.³ CO₂	Diff.	without mm.³ CO₂	with mm.³ CO₂	Diff.	
	mgm.			*per cent*			*per cent*	
Methyl (caffeine)..........	1.2	150	175	16				3
Methyl......	1.2	146	158	8				3
Methyl.....	1.2	175	186	6				
Methyl...	1.2	151	185	22				
Methyl....................	1.2	169	224	32	480	456	−5	3
Ethyl.....................	1.2	187	212	13	560	430	−23	3
Butyl.....	1.2	125	115	−8	457	405	−12	3
n-Propyl.	1.2	129	145	12	438	433	−1	3
Crotyl.	1.2	140	190	36	446	450	1	3
Allyl..	1.2	102	122	20	360	344	−5	3
Methallyl..................	1.2	116	124	7	467	470	1	3
Methoxyethyl	1.2	121	138	14	404	403	0	3
Isoamyl....................	1.2	128	164	28	435	402	−8	3
Theophylline..............	1.2	141	148	5	475	486	2	2
Urethane..................	1.2	93	92	−1	307	305	−1	3
2 Aminopyrimidine.........	3.6	116	115	−1	391	373	−5	3

Concentrations of solutions: 1.2 cc. extract; 0.4 cc. of 1.3 per cent sodium bicarbonate; 0.2 cc. of 4.0 per cent glycogen; 0.4 cc. of 0.3 per cent theobromine derivative. Total volume in each vessel made up to 2.2 cc. 95 per cent nitrogen and 5 per cent carbon dioxide. 25.0°C.

* Glycolysis reported as average of results of the number of experiments given in last column.

† Concentration given as number of mgm. present in 2.2 cc. of enzyme solution.

TABLE 2

Effects of caffeine and magnesium sulfate on glycolysis

EXP.	GLYCOLYSIS (MM.³ CO₂)							
	10 minutes				60 minutes			
	Caffeine	Caffeine MgSO₄	MgSO₄	Diff.	Caffeine	Caffeine MgSO₄	MgSO₄	Diff.
1	118	73		−34	290	257		−4
2*	155	103		−33	357	349		−2
3		73	118	−34		257	290	−11

* Average of two determinations in Exp. 2.

Concentrations of solutions: 1 cc. of extract (1 part muscle, 1.5 parts water); 0.4 cc. of 1.3 per cent sodium bicarbonate; 0.2 cc. of 4.0 per cent glycogen; 0.2 cc. of 1.0 per cent magnesium sulfate; 0.4 cc. of 0.3 per cent caffeine. 95 per cent nitrogen and 5 per cent carbon dioxide. 25.7°C.

Following these experiments, several determinations were made with (1) magnesium sulfate and (2) iodoacetate and fluoride and (3) phosphate in the

caffeine first stimulates glycolysis and then depresses. If magnesium sulfate is added, the effect of caffeine is a depressant one on glycolysis while if phosphate is added to the reaction, the effect of caffeine if one of stimulation. Determinations of inorganic phosphate show a greater amount of this substance when caffeine is present. No decision can be reached at the present time concerning the underlying mechanism nor can any final statement be made concerning the primary stimulation and secondary inhibition seen in these experiments. This change is probably associated in some way with magnesium and phosphate ions for it was shown that the presence of magnesium in the absence of added phosphate slows the rate of glycolysis when caffeine is present while the addition of phosphate increases glycolysis when caffeine is added to the reaction vessel.

The author wishes to thank Mrs. F. P. Lively for her technical aid in these experiments.

CONCLUSION

1. Caffeine and some theobromine derivatives cause a primary increase in the rate of glycolysis of glycogen to lactate followed by a secondary inhibition in extracts of frog's muscle.

2. When magnesium sulfate is added, caffeine inhibits glycolysis. When phosphate is added, caffeine stimulates glycolysis.

3. When caffeine is present, there is more inorganic phosphate present in the solutions both at the beginning and the end of the one hour experiment.

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In the experiments in which phosphate was determined, it was observed that when caffeine was present the inorganic phosphate was higher than when caffeine was not added to the extract (table 6). This difference was also observed in the samples taken at the beginning of the experiment. Control experiments were made without the presence of the muscle extract. In these experiments, no marked difference was observed in the phosphate concentration. Therefore

TABLE 5

Effects of sodium fluoride, iodoacetic acid and caffeine on glycolysis

EXP.	GLYCOLYSIS (MM ^{3} CO$_2$)			
	10 minutes		60 minutes	
	Without caffeine	With caffeine	Without caffeine	With caffeine
1	19	16	21	17
2	12	20	37	44

Concentrations of solutions: 0.8 cc. of extract; 0.2 cc. of 2.6 per cent sodium bicarbonate; 0.2 cc. of 2.7 per cent sodium fluoride; 0.2 cc. of 4.0 per cent iodoacetic acid; 0.2 cc. of 4.0 per cent glycogen; 0.2 cc. of 1.0 per cent magnesium sulfate; 0.2 cc. of 0.6 per cent caffeine; 0.2 cc. of .1 M phosphate. 95 per cent nitrogen and 5 per cent carbon dioxide. Temperature, 25.8°C. Results given as average of two determinations.

TABLE 6

Effects of caffeine on "inorganic phosphate" in glycolysis

EXP.	"INORGANIC PHOSPHATE" (MGM. PER CENT)			
	0 minute		60 minutes	
	Without caffeine	With caffeine	Without caffeine	With caffeine
1	23.8	38.1	31.8	38.9
			31.8	38.1
			32.3	38.8
2	27.5	37.5		
	33.8	38.8		
3	28.7	37.7	28.1	35.6
	28.7	37.7		
4	28.7	37.7	28.7	40.5
	28.7	37.7	30.0	40.5

Concentrations of solutions: 1.0 cc. of extract; 0.2 cc. of .1 M phosphate; 0.2 cc. of 4.0 per cent glycogen; 0.4 cc. of 0.4 per cent caffeine; 0.4 cc. of sodium bicarbonate. 0 minute determinations were made immediately after spilling over glycogen into tubes.

caffeine is aiding in the augmentation of free phosphate when the enzyme mixture is present. This reaction is very fast for no marked changes were detected during the time of the experiments from the beginning values (table 6).

DISCUSSION OF RESULTS. These experiments demonstrate that caffeine and some theobromine derivatives have an effect on glycolysis of glycogen to lactate in muscle extracts. In the system, bicarbonate + glycogen + muscle extract,

of cholinesterase was that of Ammon with slight modification (1). The final reaction volume was 4 cc. and the final acetylcholine concentration 0.015 M. The vessels were equilibrated with 95 per cent nitrogen and 5 per cent carbon dioxide. After thermal equilibrium at 38°C. was reached, the vessels were tipped and readings taken at 10 minute intervals. Results are expressed in absolute terms, i.e., cmm. carbon dioxide/gm. of tissue/30 minutes. The tissues were prepared as follows: Homogenates of brain, nerve, and muscle were made by grinding with washed sand in 0.03 M sodium bicarbonate. Cerebral cortex was the source of brain samples, triceps and gastrocnemii were used for muscle determinations, and the nerves used were the brachial plexus and sciatic nerves. The main compartment of the vessels contained: nerve, 3.0 cc. of a 1:10 homogenate and 0.5 cc. of water; brain, 1 cc. of a 1:10 homogenate, 2 cc. of 0.03 M sodium bicarbonate, and 0.5 cc. of water; muscle, 3 cc. of a 1:2 homogenate and 0.5 cc. of water. To the side arm of the above vessels was added 0.5 cc. of 0.03 M sodium bicarbonate containing 0.12 M acetylcholine.

RESULTS. Symptomatology: Observations were made on 20 cats which received a daily intra-muscular dose of 1 mgm./kgm. of DFP in peanut oil, until signs of severe intoxication were manifest. These consisted of ataxia, extreme muscular weakness, and generalized fasciculations. The number of daily doses required to achieve this varied from 2 to 6, (average 4). At this time DFP administration was discontinued. The course of the poisoning was as follows: fasciculations which were evident during administration gradually subsided and disappeared entirely by the 3rd to 4th day[2] after the last dose of DFP, generalized weakness which varied from complete prostration to ataxic gait persisted until the 2nd to 15th days, (average 7th). Subsequent to the disappearance of obvious weakness, and at a time when the animal appeared grossly normal, it was possible to elicit and intensify a syndrome of weakness by means of forced exercise. This state of fatiguability lasted for from 5 to 33 days, (average 12). Fatiguability was evidenced in various ways such as the development of a generalized motor weakness and ataxia after exercise, often progressing to complete prostration, occasionally dyspnea, and fatigue on mastication.

A second phase of the syndrome became apparent subsequent to the disappearance of the generalized weakness. This consisted of a recurrence of the weakness, chiefly confined to the hind legs and varying in severity. The onset of this occurred anywhere from the 5th to the 12th day, (average 9th) and was independent of the site of injection. The course of this damage has been followed in several cats, all of which made complete recoveries by the 21st to 147th day after the last dose of DFP. The reflexes in the limbs were normal and gross atrophy was not noted.

NEUROMUSCULAR FUNCTION. Experiments were performed on 16 cats which had received 3 to 5 daily intramuscular doses of 1 mgm./kg. of DFP. At varying intervals after poisoning, the following were studied: (1) the threshold of response to the close intra-arterial injection of acetylcholine, and (2) the ability of the muscle to maintain a tetanus induced by nerve stimulation.

Similar experiments performed on 5 normal cats showed: (1) a threshold of response to acetylcholine of 5 to 10 μg./kg., and (2) a well maintained tetanus to nerve stimulation for longer than 90 seconds.

[2] Henceforth refers to days after the last dose of DFP.

THE EFFECT OF CHRONIC POISONING WITH DI-ISOPROPYL FLUOROPHOSPHATE ON NEUROMUSCULAR FUNCTION IN THE CAT[1]

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Di-isopropyl fluorophosphate (DFP) has been shown to inactivate irreversibly the cholinesterases of serum and tissues (1). In an early study on the general pharmacology of DFP, Modell, et al. found that cats which had received large doses of this agent frequently developed a protracted syndrome of muscular weakness. This persisted for a period of 4 to 17 days, and in 1 animal, after repeated doses of DFP, lasted as long as 5 months (2). Koelle and Gilman investigated the chronic toxicity of DFP in dogs, monkeys, and rats, (3). They noted that the repeated administration of sub-lethal doses of DFP to dogs resulted in the development of hind leg weakness which proceeded to almost complete paralysis. Subsequent to the withdrawal of DFP some functional improvement occurred but complete recovery was never noted. The doses of DFP given to rats and monkeys did not elicit any symptoms of neuromuscular abnormalities.

The present report is concerned with the results of a study on the prolonged neuromuscular disturbances which follow the administration of repeated large doses of DFP in the cat.

METHODS *Nerve-muscle preparation* Normal and experimental cats were anesthetized by the intra-peritoneal injection of 0.5 cc /kg. of "Dial" (Ciba). The gastrocnemius muscle was freed by dividing and elevating the Achilles tendon. Using a holder described by Wolff and Cattell (4), the proximal end of the tibia was fixed by a steel pin and the tendon wired vertically to an isometric lever Recordings were made on a smoked kymograph drum. The popliteal artery was exposed for injection and no attempt was made to ligate branches of this artery going other than to the gastrocnemius. Enclosed silver electrodes were fixed in place on the distal end of the cut sciatic nerve. Injections made into the popliteal artery with a #26 needle were as rapid as possible and during injection the artery was temporarily occluded above Drugs to be injected were dissolved in distilled water so as to have a constant volume of injection of 0 1 cc./kg. A DuMont variable frequency stimulator, type 210, was used, providing a stimulus of constant frequency, amplitude and form, which was submaximal in strength The frequency of stimulation was usually 18.5 per second. In certain experiments maximal break shocks were used, delivered from an inductorium by an interruptor.

Cholinesterase determination The manometric method employed for the determination

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isotonic recording as illustrated in figure 2. These findings are similar to those reported by Brown (6) for denervated mammalian striated muscle. In contrast

FIG. 1. ISOMETRIC RESPONSES OF GASTROCNEMIUS MUSCLE
A: Normal cat, 5 ugm./kg. of acetylcholine intra-arterially. *B:* Cat #31, 8 days after DFP, 10 and 24 ugm./kg. of acetylcholine i.a. *C:* Denervated muscle, (11 days), 0.001 and 0.01 ugm./kg. of acetylcholine i.a. *D:* Cat #30, 10 days after DFP, 6 mgm./kg. of KCl i.a.

FIG. 2. ISOTONIC CONTRACTION OF GASTROCNEMIUS IN RESPONSE TO ACETYLCHOLINE. I.A.
Record at left: normal cat. Record at right: cat #62, 6 days after DFP

to completely denervated muscle, increasing the dose of injected acetylcholine augments rather than diminishes the "quick" phase.

In chronically poisoned animals as in chronically denervated animals, the intra-

The results obtained from the chronically poisoned animals are summarized in table 1. These consisted of an increased sensitivity of the muscle to acetylcholine, a prolongation of the contractile response produced by acetylcholine injection, and an inability of the muscle to maintain a tetanus during indirect stimulation.

RESPONSES TO ACETYLCHOLINE   The increased sensitivity to acetylcholine was in evidence from the 1st to the 10th day after poisoning. These animals responded to doses of 0.25 to 1.0 μg./kgm. of acetylcholine intra-arterially. A

TABLE I

The effect of daily intramuscular doses of DFP on neuromuscular function in the cat

CAT NO	DAYS AFTER DFP	TOTAL DFP DOSE	SYMPTOMS				ACETYL-CHOLINE THRESHOLD	MAINTENANCE OF TETANUS
			Fas *	GW†	Fat ‡	HLW§		
		mg/kg					*μg/kg.*	*(Sec)*
37	1	4	++	++++	−	−	1	−
39	2	3	+	++++	−	−	1	60−
16	4	3	+	++++	−	−	0 25	30−
25	4	3	0	++++	−	−	1	60−
28	6	3	0	+	+++	+	0 25	30−
32	6	3	0	++++	−	−	1	30−
62	6	3	0	++	+++	++	1	90
31	8	3	0	+	+++	++	1	90+
30	10	3	0	++	++++	++	5	90+
9	14	3	0	++	+++	++	1	60−
29	15	3	0	0	++	+	5	90
18	18	3	0	0	++	+	5	90−
14	27	4	0	0	0	++	5	90+
10	31	5	0	0	+	+	5	90−
15	33	3	0	0	0	±	5	90+
49	45	3	0	0	0	0	5	90+

* Fasciculations

† Generalized weakness

‡ Fatigability

§ Hind leg weakness

similar increase in sensitivity to acetylcholine has been noted after the acute administration of DFP (5).

In animals 4 to 18 days after poisoning the configuration of the contractile response to the close intra-arterial injection of acetylcholine resembled that of chronically denervated mammalian muscle. This contractile response, at threshold doses of acetylcholine consisted of a twitch-like contraction which was slow to relax. With higher doses of acetylcholine, a second contractile response appeared which resembled in duration and configuration the "slow" component of denervated mammalian muscle and which increased as the dose of acetylcholine became larger. The total duration of such a contractile response, recorded isometrically, was as long as 22 seconds (fig. 1). This response was intensified with

shown in table 2. The regeneration of muscle cholinesterase is the most rapid, being complete in 2 weeks. By 1 month the brain cholinesterase has reached 69 per cent and the nerve 85 per cent of their control values. Serum cholinesterase regeneration was followed in a few animals and conformed to previously reported data (7).

DISCUSSION. The appearance of generalized fasciculations, ataxia, and weakness during and immediately after DFP treatment coincides with an extreme reduction of muscular and nervous tissue cholinesterase as has been noted in the
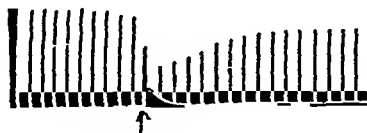
FIG. 4. EFFECT OF ACETYLCHOLINE, 1 UGM/KG I.A., DURING STIMULATION OF SCIATIC NERVE WITH SINGLE MAXIMAL BREAK SHOCKS, IN CAT 4 DAYS AFTER DFP

TABLE II

Cholinesterase activity after poisoning by DFP

DAYS AFTER DFP	BRAIN		NERVE		MUSCLE	
	Cmm CO_2/ gm /30 min	Per cent of normal	Cmm CO_2/ gm /30 min	Per cent of normal	Cmm CO_2/ gm /30 min.	Per cent of normal
1	170 (5)*	16 9	4 (4)*	3	2 (5)*	6
2	250 (4)	24 9			14 (4)	39
4 5			39 (15)	30		
5.5	280 (12)	27 9			17 (11)	47
16	316 (5)	31 5	72 (4)	55	43 (5)	120
31	692 (6)	69	110 (6)	85	55 (6)	153

* Refers to number of cats

acutely poisoned animal The symptomatic recovery of the chronically poisoned animal parallels the regeneration of cholinesterase in the aforementioned tissues. This would suggest that the syndrome of weakness and fatiguability results from an accumulation of acetylcholine at the motor end plate. However, the protracted fatiguability and more especially the hind leg weakness cannot be correlated entirely with a lowered tissue cholinesterase.

The inability of the muscle to maintain a tetanus is attributed to a failure of neuromuscular transmission and also appears to be related to the lowered cholinesterase activity of the muscle DFP has no known effect on the contractile mech-

arterial injection of KCl evokes a contractile response like that produced by acetylcholine (fig. 1).

In one cat, in which the sciatic nerve was sectioned aseptically 17 days previously, and which was chronically poisoned by DFP 4 days prior to the experiment, the response of the muscle to the close intra-arterial injection of acetylcholine was tested. This animal showed the same threshold and configuration of response as untreated, chronically denervated controls.

RESPONSES TO NERVE STIMULATION. The failure of the muscle to maintain a tetanus when the nerve was stimulated submaximally, occurred from the 1st to the 6th day after poisoning (fig. 3); recovery after 30 days was usual. In 3 cats the inability to maintain a tetanus was noted on the 14th, 18th and 31st days.

The response of the gastrocnemius to single maximal nerve stimuli was tested in 4 animals, 2 on the 2nd, 1 on the 3rd, and 1 on the 4th day after poisoning.
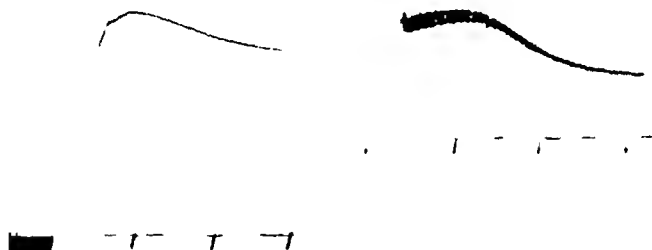
FIG. 3. ISOMETRIC CONTRACTION OF GASTROCNEMIUS IN RESPONSE TO TETANIC STIMULATION OF SCIATIC NERVE

Record at left normal cat. Record at right. cat #9, 14 days after DFP Frequency of stimulation 18 5/sec Time interval. 30 sec.

These animals had a spinal transection at L_1 under preliminary ether anesthesia. The branches of the popliteal artery going other than to the gastrocnemius were ligated. At a constant rate of 1 stimulus in 12 seconds, no fatigue was noted. However, the close intra-arterial injection of acetylcholine during stimulation, caused a protracted depression of the response in 1 animal 2 days after DFP. In another cat 2 days after poisoning, potentiation of the responses to succeeding nerve stimuli occurred after small doses of acetylcholine. Similar findings have been reported after the acute administration of DFP (5). In the cats tested 3 and 4 days after poisoning, a prolonged contractile response to the close intra-arterial injection of acetylcholine occurred together with a depression of the response to single nerve shocks (fig 4).

CHOLINESTERASE. At varying intervals after poisoning by DFP, tissues were examined for their cholinesterase activity. A total of 32 chronically poisoned cats were used. Normal values were determined on 20 cats. The results are

probable that the syndrome of chronic DFP poisoning is a result of a decreased cholinesterase activity at the neuromuscular junction which, when prolonged, results in an injury of the muscle which resembles that following section of the motor nerve. These results emphasize that the chronic administration of DFP produces a secondary change in neuromuscular function which is not present in the acutely poisoned animal.

SUMMARY

1. The daily intramuscular injection of DFP in cats, resulted in the appearance of ataxia, extreme muscular weakness, and generalized fasciculations. Subsequent to this it was possible to demonstrate weakness and fatigableness by means of forced exercise. Finally, a recurrent weakness appeared in the hind limbs and lasted for as long as 147 days.

2. The cholinesterase activity of brain, nerve, and muscle has been measured at intervals after poisoning. The symptomatic recovery of the poisoned cat approximates the regeneration of the tissue esterases.

3. The response of the gastrocnemius muscle to the intra-arterial injection of acetylcholine was altered in the poisoned animals. This consisted of an increased sensitivity to acetylcholine and a prolongation of the contractile response resembling that seen in denervated muscle.

4. The muscle of the poisoned cat manifests an impaired ability to maintain a tetanus as compared to normal animals.

5. It is concluded that the syndrome described results from the extreme reduction of the muscle and nervous tissues cholinesterase by DFP. The changes in the response of the muscle to acetylcholine following protracted inactivation of cholinesterase, suggest the development of an injury at the myoneural junction resembling that associated with denervation.

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anism of mammalian skeletal muscle. This is best evidenced by the fact that the intra-arterial injection of DFP does not affect the response of the completely curarized, directly stimulated tibialis anticus of the cat, (5); in addition, it does not evoke a contractile response in denervated cat muscle (8). The diminished cholinesterase activity of the muscle is also thought to be responsible for the protracted depression of the response to single nerve shocks which follows the intra-arterial injection of amounts of acetylcholine which normally are not paralytic.

The increased sensitivity to injected acetylcholine which occurs in poisoned animals may be accounted for in part by an inhibition of muscle cholinesterase activity or in part by the production of a denervation-like injury.

Within the first 24–48 hours following the final DFP dose, the enhanced sensitivity of the muscle to acetylcholine is present; in contrast to this the prolongation of the contractile response is not yet in evidence. This suggests that the primary change in acetylcholine sensitivity results from a reduction of the muscle cholinesterase activity. This is further supported by the fact that the repeated intra-arterial injection of small doses of acetylcholine at this time readily renders the muscle refractory to both nerve stimulation and further acetylcholine injections. At a later stage in the chronically poisoned animal (10th to 18th day), the increased sensitivity to acetylcholine may disappear while the prolongation of the contractile response continues to be manifest.

The altered response to the close intra-arterial injection of acetylcholine resembles the response of chronically denervated mammalian muscle. The interval after poisoning before its appearance is comparable to the time required for the altered response to acetylcholine subsequent to nerve section. That the denervation-like effect is incomplete, is evidenced by the fact that the response to nerve stimulation is not abolished. In addition, suitable doses of acetylcholine elicit first the rapid twitch-like effect followed by a slow contracture-like response. Definitive evidence as to the production of a true denervation by this means must await careful histologic study.

The denervation-like effect after poisoning by DFP may result from a prolonged inhibition of cholinesterase with a resultant accumulation of endogenous acetylcholine, or from some additional action of DFP on the muscle apart from its anti-esterase property. To test these possibilities, similar experiments were performed on cats chronically poisoned with another anti-cholinesterase, namely, physostigmine. These animals were injected intramuscularly at hourly intervals for a period of 8 hours daily over the course of 5 days and protected by atropine. The appearance of these animals was similar to that of cats chronically poisoned with DFP. The response of the muscle to the intra-arterial injection of acetylcholine was tested 3 to 4 days after the last dose of physostigmine. The response obtained was again similar in configuration to that of denervated muscle.

It has been reported that chronic poisoning in cats following large single doses of DFP, can be prevented by pre-treatment with rather small doses of physostigmine which serves to protect the cholinesterase from inactivation by DFP, (9). Although some additional action of DFP cannot be excluded entirely, it appears

FURTHER EXPERIMENTS IN AN ATTEMPT TO LOCATE THE SITE OF THE EMETIC ACTION OF THE DIGITALIS GLYCOSIDES

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Nausea and vomiting occurring in a patient receiving digitalis has long been regarded as a signal of approaching or actual overdosage of the drug. The site of the stimulation for this vomiting has been the object of many investigations resulting in conclusions among which there has been no general agreement.

Much of the existing evidence is of a negative nature. Thus, emesis has been shown to persist after removal of the gastro-intestinal tract (1), after hepatectomy (2, 3), and after complete cardiac denervation (4). Some evidence has been brought forth that the direct action of digitalis in the medulla is not responsible for this emesis (5, 6).

Among the conclusions of a positive nature have been those of Haney and Lindgren (7) who concluded that emesis most likely resulted from a direct stimulation of the medullary vomiting center. The preliminary reports of the writer (8, 9, 10, 11) have all been in agreement with this conclusion. Contrary to the findings of this author, Hanzlik and Wood (12) decided from their observations in pigeons that the liver was probably the primary site of emetic stimulation.

The present investigation is a continuation of previously reported work (8, 9, 10, 11) undertaken in an attempt to secure further information as to the site of the origin of the emetic response.

EXPERIMENTAL METHODS. As in previous publications the experiments to be reported upon in this paper have been performed upon cats and female dogs. After re-examining the data from the preliminary experiments, repeating some of the procedures and extending some of the surgical denervations, the final plan devised was to ascertain in chronic experiments what effect sectioning of all visceral afferent neural pathways leading to the brain would have upon the initiation of emesis by glycosides.

TYPES OF PROCEDURES UTILIZED. The exact details of the surgical procedures are unimportant. In general, the dorsal spinal roots from T2, T1, or C8 to C5 or C3 were cut and the spinal cord was transected just below the lowest pair of roots cut, the exact location of such transections being confirmed at subsequent postmortem examinations.

Following recovery from this operation and at variable periods of time as indicated in table 3, bilateral vagotomy was performed. The procedure used for vagotomy was exposure of the nerves using local infiltration of procaine for anesthesia, followed usually by the nerve being blocked with nupercaine solution. Emesis tests were made within an hour and then

diaphragmatic tone and movement or in the case of panting may cause a conflict between the respiratory center and the center controlling diaphragmatic movement.

TABLE 1

Postoperative reduction in emetic reactivity

TRANSECTION LEVEL AND NEUROTOMIES	SUBSTANCES INJECTED	AMOUNTS IN mg. per kg.		POSTOPERATIVE INTERVAL	NO. OF VOMITING ACTS IN 1 TEST	LATENCY IN MINUTES
		Pre-op.	Postop.			
Thoracic 1, Dog #3	Strophanthidin, I.V.	0.06	—		+	3
			0.07	4 days	Nausea	2
			0.08	7 days	Nausea	1
			0.08	10 days	+	12
			0.07	11 days	+	15
			0.07	12 days	+	1½
Thoracic 2, Dog #1	Strophanthidin, I.V.	0.06	—		+	6
			0.08	2 days	Nausea	4½
			0.09	3 days	Nausea	3
			0.09	5 days	Nausea	5½
			0.09	6 days	—	—
			0.10	10–17 days	+	1–7
			0.10	21 days	+	4½
Dor. Rhizot., C3–C7, Inclusive, Dog #2	Strophanthidin, I.V.	0.04	—		+	5
			0.04	6 days	—	—
			0.04	11 days	Nausea	7
			0.04	19 days	—	—
			0.05	21 days	+	6
Thoracic 3, Cat #1	Strophanthidin, I.M.	0.20	—		+	30
	Ouabain, I.M.	0.05	—		+	?
			0.05	5 hours	—	—
			0.05	5½ hours	—	—
			0.05	6 hours	+	52
	Strophanthidin, I.M.		0.05	24 hours	Fatal	5
Thoracic 2*	Strophanthidin, I.M.	0.20	—		+	20
	Ouabain, I.M.	0.05	—		+	18
			0.05	4½ hours	—	—
			0.05	5 hours	—	—
			0.05	5¾ hours	Nausea	65
			0.05	6 hours	Fatal	10

* Seventeen days after low intrathoracic vagotomy.

When the animals had recovered from the operative procedures, as indicated by their desire for food, the following stages in the emetic reaction were noted before the animal was able to participate in emesis as seen pre-operatively: (1) nausea, indicated by the characteristic licking, salivation, repeated swallowing and restlessness; (2) rhythmic contractions

a segment of each nerve was removed. In some instances, in animals surviving a long time, vagotomies were repeated (e g. Cat #1, Table 3).

Completion of the entire surgical procedure for complete visceral deafferentation as outlined above was not possible in all instances. However, certain observations could be made from these incomplete preparations, and they are grouped together in Table II and designated as "Partial Collective Deafferentation".

CARE OF THE ANIMALS. Due to the nature of the surgical procedures certain precautions had to be taken and technics had to be developed to keep the animals in good condition. In the cat it was necessary to prevent closure of the glottis when the vagotomy was performed. This was accomplished by the removal of a portion of one thyro-arytenoid cartilage prior to vagotomy. In cats observed for two years there was no restoration of this cartilage.

Following the rhizotomy the animals were placed in thermostatically controlled cages so regulated as to maintain rectal temperature between 37.5 and 39.0°C. The beds in these cages were kept soft and clean; spontaneous evacuations were prevented by daily warm water enemas and by frequently emptying the bladder with manual pressure.

Fluids were given by mouth, vein or rectum as indicated. Milk and meat were fed when the animals could eat satisfactorily. Care had to be taken in the cats who had had the partial thyro-arytenoidectomy so that food and excess saliva were not aspirated. This required careful feeding with a pipette. Vagotomized dogs were not fed by mouth. Where oral feedings were precluded, the feedings were supplemented by aminoids by rectum.

MATERIALS USED AND METHODS OF ADMINISTRATION. The pure glycosides used were ouabain, thevetin, k-strophanthoside dissolved in water with 7 to 8 per cent alcohol, and the lanatosides A, B, and C dissolved in water with 25 per cent ethanol and 10 per cent glycerol. Also studied for various purposes were strophanthidin, apomorphine hydrochloride and nicotine hitartrate.

In order to test the action of more than one glycoside in an animal, it was necessary to shorten the duration of action of the drug and prevent serious toxic effects; e.g., myocardial necrosis, well known to occur in cats given toxic but not necessarily lethal amounts of glycoside (13, 14). Consequently smaller doses were given and advantage was taken of the rapid elimination of strophanthidin (15, 16.) Thus, since a single emetic dose seldom exceeded 0 10 milligram per kilogram, it was largely, if not entirely, eliminated or deactivated within one hour after intravenous injection. In the unoperated dog and in the spinal cats the minimal emetic dose could be determined with considerable sharpness. All injections were standardized at the rate of 0.01 milligram per kilogram per second.

The routine developed for drug administration was to inject one-half of an emetic dose of the glycoside either intravenously or intramuscularly. In an hour one-half emetic dose of strophanthidin was given by vein. If an emetic response did not occur within 30 to 60 minutes, a small additional quantity of strophanthidin was injected, which usually induced vomiting.

This method enabled one to gain a good idea of the persistence of emetic action of individual glycosides because progressively larger amounts of strophanthidin were required to induce emesis during the time the glycosides were being eliminated. It could also be shown that the increased sensitivity of the vomiting mechanism established by any glycoside could be maintained at a surprisingly constant level by a small daily injection of the glycoside. Strophanthidin used in this manner was also of value in observing the influence on emesis of anesthesia, surgical procedures, alterations of body temperature and repetition of tests.

ALTERATIONS OF THE EMETIC RESPONSE. In experiments of this type the characteristics of the emetic response as seen in the normal animal may be lacking following the operative procedures, particularly vagotomy. Vomiting may be temporarily suppressed by the following mechanisms: (1) tachycardia or excitement, (2) bradycardia, (3) hypotension, (4) anything which might interfere with the participation of the diaphragm in the emetic response, such as: (a) panting, (b) vagotomy and (c) phrenotomy, all of which may affect

TABLE 2—*Concluded*

OPERATIVE PROCEDURE	SUBSTANCES INJECTED	AMOUNTS	POST-OPERA-TIVE INTER-VAL	NUMBER OF VOMITING ACTS IN 1 TEST	LA-TENCY
		mg./kg.	*days*		*min.*
Cord Transection at T-1, Cat 5 (3.0 kg.)	Strophanthidin	0.06	7	N++	11
Right Vagotomy†	Strophanthidin	0.08	264	+	12
Cord Transection at T-L, C8 + T-1 Ventral + Dorsal Rhizotomy, Cat 4 (2.9 kg.)	Strophanthidin	0.10	37	+	5
Bilateral Vagotomy*, Cat 4, (2.0 kg.)	Lanatoside B	0.10	46	N+	2
	Strophanthidin	0.10	48	+++	7
Cord Transection at T-2, Cat 3 (2.5 kg.)	Strophanthidin	0.14	24	+	10
Bilateral Vagotomy*	Lanatoside B	0.12	35	+	8
	Strophanthidin	0.10	42	+++	7
Dorsal Rhizotomy C3-C7 Inclusive, Dog 8 (8.4 kg.)	Strophanthidin	0.05	21	+	6

* Bilateral Vagal Block several days to weeks later. Survival time ranged from a few hours to, in most cases, weeks or months.

† Mydriasis hereafter associated with glycosidic emetic action and not altered by right superior cervical ganglionectomy.

‡ Number of attacks of nausea.

§ Bilateral nupercaine vagal block followed by injection of test substance within an hour.

of the diaphragm, typically coordinated with movements of the head in the vertical plane; and (3) final flexion and extension of the head with the mouth opening. Typical retching could not occur (17).

An emetic reaction to any agent was considered to have been induced in the animals after operation when stage (1) alone was present and typical. Stage (2) was the more usual reaction in these chronic preparations.

EXPERIMENTAL RESULTS. The data presented in this and other recent reports have been compiled from observations on one hundred and twenty animals upon whom over one thousand emesis tests were made. The observations on sixty-three animals form the basis of this report.

The author has previously contended that it was not necessary to increase the amount of glycoside injected post-operatively above that amount needed pre-operatively to induce emesis. Table 1 contains some data that would verify this statement. However, in some of the animals indicated in table 3, the post-operative amount of glycoside injected to induce emesis was larger than needed pre-operatively. In general, these amounts were larger than necessary, and it is felt that if an attempt was made to determine carefully pre-operative and post-operative M.E.D.'s in dogs and cats free from excitement and postoperative complications, there would be no significant difference.

TABLE 2

Partial collective visceral deafferentation

OPERATIVE PROCEDURE	SUBSTANCES INJECTED	AMOUNTS	POST-OPERATIVE INTERVAL	NUMBER OF VOMITING ACTS IN 1 TEST	LATENCY
		mg./kg.	days		min.
Total Sympathectomy, Dog 3 (7.7 kg.)	Strophanthidin	0.07	14	++	4
Total Sympathectomy, Dog 2 (10.5 kg.)	Strophanthidin	0.09	30	+	5
	Lanatoside C	0.05	40	—	—
	Strophanthidin	0.07	40	+	8
Total Sympathectomy, Dog 1 (9 3 kg.)	Strophanthidin	0.08	15	+	6
	Lanatoside A	0.05	21	—	—
	Strophanthidin	0.04	22	++	8
Bilateral Vagal Block*§, Dog 1 (9.6 kg.)	Strophanthidin	0.06	23	+	6
	Apomorphine HCl	0.02	23	+	3
Cord Transection at T-1, Dog 7 (5.6 kg.)	Strophanthidin	0.08	6	+	6
Bilateral Vagotomy*, Dog 7 (5.5 kg.)	Ouabain	0.04	7	—	—
	Strophanthidin	0.06	8	+++	2
	R-strophanthoside	0.06	8	++	32
Cord Transection at T-1, C8 + T-1 Ventral + Dorsal Rhizotomy, Dog 6 (10.0 kg.)	Strophanthidin	0.08	11	+	12
Bilateral Vagal Block*§, Dog 6 (8.3 kg.)	Strophanthidin	0.09	27	+	2
	Lanatoside B	0.06	27	—	—
	Strophanthidin	0.04	27	+	2
	Apomorphine HCl	0.004	27	+	3
Cord Transection at T-1, C8 + T-1 Dorsal Roots Cut, Dog 4 (6.4 kg.)	Strophanthidin	0.10	16	+	9
	Lanatoside A	0.05	20	—	—
	Strophanthidin	0.05	20	N+++‡	3
Cord Transection at T-1, C8 + T-1 Dorsal Roots Cut, Dog 5 (6.5 kg.)	Strophanthidin	0.08	11	+	4
	Lanatoside A	0.04	21	—	—
	Strophanthidin	0.04	21	+	4
	Lanatoside C	0.03	29	—	—
	Strophanthidin	0.04	29	+	12
Cord Transection at C8, C7–C3 Inclusive Dorsal Roots Cut, Cat 2 (2.6 kg.)	Strophanthidin	0.12	25	++	3
Bilateral Vagotomy*, Cat 2 (2.0 kg.)	Strophanthidin	0.12	44	++	4
Cord Transection at T-1, C8–C4 Inclusive Dorsal Roots Cut. Cat 1 (2.9 kg.)	Strophanthidin	0.08	3	+	3
	Strophanthidin	0.06	8	N++++	5

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TABLE 3
Complete visceral deafferentation

DATE OF CORD TRANSECTION AND DORSAL RHIZOTOMY	PROCEDURES AFFECTING VAGI	SUBSTANCES INJECTED	PRE-OPERATIVE M.E.D.	POSTVAGOTOMY DOSAGE (mg/kg) & ANIMAL'S WT. IN KG. ON DATE DRUG ADMINISTERED	NO. OF VOMITING ACTS IN ANY INDIVIDUAL TEST	LATENCY	SURVIVAL TIME Cord Transection	SURVIVAL TIME Post Vagotomy
			mg./kg.			min.	days	days
Dog #3 (6.8 kg.), April 1	Bilateral Vatotomy, April 9	Strophanthidin	0.09	0.12† Apr. 9 (5.7 kg.)	++++	3	9	1
Dog #2 (7.8 kg.), April 23	Bilateral Vagotomy, May 6	Strophanthidin / Lanatoside C / Ouabain	0.08 / 0.08	0.12† May 6 (6.6 kg.) / 0.03 May 7 (6.3 kg.)	+++ / N+++	4 / 2	14	1
Dog #1 (3.7 kg.), December 23	Right Vagotomy, January 8 / Left Vagotomy, January 10	Strophanthidin / Lanatoside C / Apomorphine HCl	0.06 / 0.08 / 0.01	0.06 Jan. 9 (3.3 kg.) / 0.10† Jan. 10 / 0.01 Jan. 12 (3.0 kg.)	N+++ / ++ / +++	4 / 22 / 3	22	4
Dog #4 (5.5 kg.), May 22	Bilateral Vagal Block, May 29 / Right Vagotomy / Left Vagal Block, June 4 / Left Vagal Block, June 9 / Left Vagotomy, June 16	Strophanthidin / Ouabain / Strophanthidin* / Lanatoside C / Lanatoside A / Strophanthidin / Lanatoside C / Thevetin / Lanatoside B / K-strophanthoside / Apomorphine HCl	0.06 / 0.04 / 0.06 / 0.08 / 0.06 / 0.08 / 0.22 / 0.10 / 0.04 / 0.02	0.02 May 29 (5.2 kg.) / 0.02 May 29 / 0.08 June 6 (5.0 kg.) / 0.20 June 9 (4.7 kg.) / 0.04 June 11 (4.5 kg.) / 0.09 June 16 (4.5 kg.) / 0.32 June 21 (4.5 kg.) / 0.12† June 23 (4.4 kg.) / 0.04 June 24 (4.3 kg.) / 0.02 June 25 (4.1 kg.)	+ / N+++ / +++ / N+++ / +++ / N+++	4 / 3 / 14 / 6 / 2 / 4 / 26 / 13 / 6	41	16
Cat #1 (3.0 kg.), June 19	Left Vagotomy	Strophanthidin	0.07	0.07 July 3 (2.4 kg.)	+	3		

	Strophanthidin	0.06 July 16	N+++	5	
Right Vagal Block, July 16	Strophanthidin	0.07 July 29	N++++	3	
Right Vagotomy, July 28	Ouabain }*	0.03 July 30 (1.8 kg.)		9	
	Strophanthidin }	0.04 July 30	++	10	
	Ouabain	0.06 Aug. 12 (1.7 kg.)	++		
	Lanatoside C }*	0.04 Aug. 27 (1.9 kg.)		4	
	Strophanthidin }	0.04 Aug. 27	+++	3	
	Strophanthidin	0.04 Aug. 28	+++	5	
	Strophanthidin	0.04 Aug. 30 (1.8 kg.)	N+++	3	
	Strophanthidin	0.07 Sept. 24 (1.9 kg.)	N		
Bilateral Vagotomy, Sept. 23	K-strophanthoside }*	0.06 Oct. 6 (1.8 kg.)		4	
	Strophanthidin }	0.04 Oct. 6	N+	2	
	Apomorphine HCl }**	0.08 Oct. 23 (1.9 kg.)	++	7	
	Strophanthidin }	0.06 Oct. 23			
	Apomorphine HCl }**	0.07 Oct. 29	+++	5	
	Strophanthidin }	0.06 Oct. 29	++	9	
	Nicotine	1.0 Aug. 5 (2.2 kg.)			413 275

* Synergistic action of bracketed pairs of these agents shown.
** The last two tests on Cat #1 suggested the possibility of apomorphine producing sensitization to the genin.
† Postvagotomy doses (Dogs #1-4) were larger than necessary as shown by the reactions.

PARTIAL COLLECTIVE DEAFFERENTATION. Table 2 contains the results of thirteen preparations that were not carried to complete deafferentation. Even a casual inspection reveals that none of the procedures employed prevented the production of emesis upon injection of an emetic dose of glycoside.

COMPLETE VISCERAL DEAFFERENTATION. The data from four dogs and one cat that were carried satisfactorily to complete deafferentation are presented in table 3. Here again emesis could be produced despite the extensiveness of the denervation procedures.

Certain individual reactions within these groups are of interest. Apomorphine was administered to two dogs (shown in table 2) and one cat (shown in table 3) to confirm the normal reaction to that drug. In the cat apomorphine almost certainly caused an increased sensitivity to strophanthidin since after the apomorphine a subemetic dose of that genin produced nausea and vomiting.

In several dogs and cats emetic responses following vagotomy failed to involve the diaphragm until several weeks postoperatively when emesis again included movement of the diaphragm.

Cat #5 (table 2) and cat #1 (table 3), the former with vagi intact and the latter with vagi sectioned, both showed hyperpnea (respiratory rate above 300 per minute) as a result of strophanthidin injections. Hyperpnea also appeared when nicotine was administered to the latter cat before strophanthidin was injected.

Two to three weeks after sectioning of the vagosympathetic trunks in cat #1 (table 3) mydriasis appeared following the administration of an emetic agent. When the dilatation attained a maximum in any given test, the emetic response seldom failed to be completed. This mydriasis was also observed in cat #5 (table 2) and was not altered by a right superior cervical ganglionectomy.

DISCUSSION. Deafferentation procedures of the type utilized in this investigation are frequently subject to criticism in that the surgical denervations were incomplete or that regeneration of the nerves sectioned occurred. The author felt that the completeness of the surgical procedures presented no especial difficulties; and since postmortem examinations were made to determine the level of nerve transections, this point needed no further consideration.

Concerning regeneration of nerve fibers; regeneration of true afferent fibers in the cervical roots can be disregarded for emesis tests were made within two to four weeks after sectioning of the nerves   Moreover, during regeneration these fibers do not penetrate the pia glial membrane at the zone of entry into the spinal cord according to Paskind (18). Regeneration of intraspinal neurones after transection, such as observed by Sugar and Gerard (19) in the cat, could not complicate matters either. In two cats kept over a year there was no evidence that pain was aroused by stimulation of hind leg receptors, neither did the cats evince any sign of cerebral control of hind leg muscles. There was no indication that pain was aroused by stimulating foreleg areas affected by the rhizotomy. Lastly, the vagus can be dismissed as regards regeneration in the cat because of the early tests and later repetition of the vagotomy. Undegenerated fibers, often found in the proximal portion of severed dorsal roots, especially in the lumbosacral region in the cat and the dog, are known to be efferent (20).

Conditioned emesis was noted in only one animal, (Cat #1 Table 3). Any of the routine procedures connected with injection of strophanthidin came to be associated with nausea. The entire procedure, omitting the drugs, was carried out over a period of six months without effect on the condition nor was it affected by a long period of rest.

The fact that nicotine can prevent emesis by glycosides has been held to be evidence of the peripheral origins of glycosidic emesis (12, 13, 14, 21, 22, 23, 24). Libet and Gerard (25), however, have shown that nicotine can block conduction centrally. Therefore, prevention of emesis by nicotine does not become significant evidence for peripheral or central emetic action. The author selected four cats: a control unoperated cat, two vagotomized cats, and a completely deafferented cat (Cat #1, Table 3). Emesis could be produced in all four preparations before administration of nicotine and in none after nicotine was injected. Since nicotine frequently produces emesis itself and does not block that emesis, then the impulses for emesis by nicotine and glycosides presumably arise at different points.

These studies and other investigations by the writer indicate that glycosidic emesis can be produced extraviscerally as neither cardiac deafferentation, abdominal vagotomy, complete visceral deafferentation nor complete hepatectomy prevented emetic responses. However, these data do not permit a dogmatic statement that the emetic action of glycosides is entirely central. There are several possibilities of other sites of action that have not been completely investigated:

(1) There might be receptors in the head, such as the eye or the labyrinth, that could be the seat of stimulation with reflex glycosidic emesis resulting.

(2) The glycosides might liberate an irritative substance (in glands or other tissues) which could act peripherally or be carried centrally in the blood to cause emesis. Of the same general theme is the possibility that a cleavage of glycosides might occur to set free aglycones that might circulate and stimulate an emetic action at one or more sites. It has been shown (16, 26) that aglycones can circulate in the blood stream, and a question of similar concern has already been asked regarding the cardiac effects of glycosides (13, 14). Again, the emetic potency of one or more such cleavage products might be greater than the original glycoside (27).

(3) There may be more than one site of stimulation which causes emesis. Krayer (28) has shown that the cardiac deceleration caused by glycosides is not due to a single mechanism, and the same principle may obtain here.

(4) Finally, reflex glycosidic emesis in man under certain conditions has not been ruled out. The brief latency mentioned by Gold, et al. (29, 30) favors this, but also rapid absorption plus high emetic potency or increased central sensitivity might explain this type of response.

SUMMARY

1. In chronic experiments on a large number of cats and dogs in good post-operative condition, glycosidic emetic responses were not prevented by deaf-

ferentation of the abdominal viscera, total hepatectomy, thorough cardiac denervation and extensive collective visceral deafferentation.

(2) In cats with complete visceral deafferentation strophanthidin emesis was blocked by the injection of nicotine.

(3) The experimental evidence favors the view that glycosidic nausea and vomiting is mainly of extravisceral origin, most probably central.

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THE EFFECT OF ASCORBIC ACID IN REDUCING THE INHIBITION OF BRAIN METABOLISM PRODUCED BY PENTOBARBITAL IN VITRO[1]

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In a previous communication (1) we presented experimental evidence which indicated that pentobarbital (nembutal) and probably other anesthetics specifically inhibit the oxidative metabolism of brain *in vitro* at the flavoprotein-cytochrome b^2 stage. In this process of oxidation either hydrogen atoms or electrons are transported from the substrate to oxygen by means of a series of oxidations and reductions involving flavoprotein, the cytochromes and cytochrome oxidase, the oxidation-reduction potential increasing in a stepwise manner as one approaches oxygen (table 1) (2). Thus it seemed possible to by-pass the anesthetic block by introducing into the system some substance which could be reversibly oxidized and reduced and which had a redox potential in the proper range. In experiments *in vitro* methylene blue was found to possess such properties ($E'_0 = +0.011$ at pH 7) (1). This dye can accept hydrogen from above the block becoming reduced to leuco methylene blue. The leuco methylene blue is reoxidized by molecular oxygen and the re-formed methylene blue can again act as hydrogen acceptor. Methylene blue, however, is not a physiological substance, and although it may increase the metabolic rate *in vitro* it produces other undesirable effects (3).

A physiological substance which has a redox potential in the proper range is ascorbic acid ($E'_0 = +0.0512(4,5)$). Furthermore, this vitamin has been shown to be antagonistic to barbiturates *in vivo* (6, 7, 8).

The present communication describes the effect of ascorbic acid and of a combination of ascorbic acid and cytochrome *c* in reducing the inhibition of oxidation by brain produced by pentobarbital.

METHODS. Warburg manometers, equipped with conical vessels of about 15 ml. capacity were used for the measurement of oxygen consumption. The centre well contained 0.3 ml. 8% KOH and a roll of Whatman filter paper, Number 40, which absorbed CO_2 from the gas phase.

Whole brain of albino rats was used in all experiments. The brain was homogenized in phosphate-Locke buffer and diluted to give a concentration of about 300 mgm. brain per ml. One half ml. of this suspension in a final volume of 1.5 ml. was used in each manometer vessel.

The buffer had the following composition: NaCl, 0.13 M.; KCl, 0.002 M.; $CaCl_2$, 0.001 M.; sodium phosphate buffer, 0.03 M., pH 7.4.

[2] For the sake of brevity we have assumed that in the metabolic cycle electrons are transferred from flavoprotein to cytochrome *b*. Actually this has not been proven but there is evidence that an intermediary does exist between flavoprotein and cytochrome *c* and that this must have a redox potential in the same range as that assigned to cytochrome *b*.

The concentration of substrate used is indicated in the tables.

The concentration of pentobarbital in these experiments was 200 μgm/ml. If one as-sumes uniform distribution of anesthetic in all tissues of a rat this concentration is higher than is necessary for anesthesia, the anesthetic dose for a rat being 40 μgm/gm. or 40 mgm./ kgm. It is unlikely, however, that the drug is evenly distributed and it is probable that the concentration is higher in the brain than in other tissues (9, 10). The concentration used in these experiments *in vitro* was not the lowest concentration which would produce a signi-ficant effect, however, since concentrations as low as 38 μgm/ml. produced inhibitions in some of our experiments.

Ascorbic acid was used in a final concentration of about 0.7 mgm. per ml.

Cytochrome c was prepared from beef heart by the method of Keilin and Hartree (11).

The gas phase in all manometric experiments was air.

The temperature of the water bath was 37°C.

The manometers were shaken in the bath for 10 minutes before being closed and readings were taken at intervals of 10 or 15 min. thereafter. The duration of the experiments varied as indicated in the tables. However, since the relationship between O_2 consumption and time was a practically linear one for the experimental periods used, this variation did not affect the conclusions drawn.

TABLE 1 (2)

E'_0 values at pH 7 and 30°C

O₂.........	+0.81 v
cytochrome oxidase..	?
cytochrome a..	+0.29 v
cytochrome c..	+0.27 v
cytochrome b .... ·	−0.04 v
flavoprotein.......	−0.08 v
pyridine nucleotide....	−0.28 v
H₂...	−0.42 v
methylene blue... .. .. · .... ·	+0.011
ascorbic acid	+0.0512

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Methods of analysis. Analyses for substrates remaining at the end of the experimental period were carried out on the contents of the Warburg vessels, using the following methods.

For glucose, after the protein was precipitated with dilute tungstic acid the method of Folin and Malmros (12) was applied. A photelometer was used to measure the intensity of the Prussian blue produced. Ascorbic acid, when present, interfered with the analysis to some extent so blank determinations on ascorbic acid alone were done and this value was subtracted from those of glucose + ascorbic acid of the experiments. Values were also corrected for the reducing substances present in brain tissue at the start of the experiment. Analyses for glucose on brain alone, ascorbic acid alone and brain incubated with ascorbic acid gave values of 0.46, 0.46 and 0.90 mgm. respectively in one experiment. In another experiment the corresponding values were 0.35, 0.39 and 0.95 mgm.

In order to negate the possibility of unreliable conclusions based on determinations of glucose alone, analyses were also done in experiments with lactate as a substrate. From the results of these analyses in which ascorbic acid did not interfere the conclusions drawn were the same as those in which glucose was the substrate.

Lactic acid was determined by a modification of the method of Lehmann (13), after re-moval of ascorbic acid by copper-lime precipitation (14).

RESULTS. An examination of table 2 shows that ascorbic acid increases the oxygen consumption of brain treated with pentobarbital to a greater extent

TABLE 2

Effect of ascorbic acid on the metabolism of glucose, lactate and pyruvate by normal and narcotized brain

SUBSTRATE	CONCEN-TRATION OF SUBSTRATE M.	CYTO-CHROME c ADDED	DURA-TION OF EXPERI-MENT	O₂ CONSUMPTION (mm³)				EFFECT OF ASCORBIC ACID ON O₂ CONSUMPTION (EXPRESSED AS PER CENT)		
				CONTROL	ASCORBIC ACID	PENTOBARBITAL	ASCORBIC ACID + PENTOBARBITAL	CONTROL		PENTOBARBITAL
			min.							
glucose....	.026	—	70	162	173	78	101	+7	(+11)*	+30 (+23)*
glucose ..	.026	—	75	275	288	80	110	+5	(+13)	+31 (+30)
glucose....	.013	—	80	221	263	109	161	+19	(+42)	+48 (+52)
glucose ..	.013	—	80	265	322	101	148	+22	(+57)	+47 (+47)
glucose ..	.013	+	75	228	321	107	246	+41	(+93)	+130 (+139)
glucose ..	.0065	+	75	238	331	111	255	+39	(+93)	+130 (+144)
glucose ...	.0032	+	75	228	360	114	264	+58	(+132)	+131 (+150)
glucose....	.013	+	80	238	279	119	199	+17	(+41)	+67 (+80)
glucose	.0065	+	75	243	304	122	207	+25	(+61)	+70 (+85)
glucose ..	.0065	+	75	248	321	133	195	+30	(+73)	+47 (+62)
glucose...	.0065	+	75	238	300	128	210	+26	(+62)	+64 (+82)
glucose..	.0065	+	75	232	292	119	169	+26	(+60)	+42 (+50)
glucose ...	.0065	+	75	237	305	99	188	+29	(+68)	+90 (+89)
glucose	.0065	+	75	247	293	119	185	+19	(+46)	+55 (+66)
glucose .	.0065	+	75	226	288	121	232	+27	(+62)	+92 (+111)
glucose .	.0065	+	75	200	255	100	245	+28	(+55)	+145 (+145)
glucose	.0065	+	75	200	258	107	176	+29	(+58)	+65 (+69)
pyruvate	.026	—	80	251	294	155	205	+17	(+43)	+32 (+50)
pyruvate	.026	—	75	198	200	117	160	0	(+2)	+37 (+43)
pyruvate	.026	—	80	303	293	240	250	−3	(−10)	+4 (+10)
pyruvate	.026	—	60	259	248	96	133	−4	(−11)	+39 (+37)
pyruvate .	.026	—	65	273	289	149	250	+6	(+16)	+40 (+101)
lactate	.013	—	120	468	355	219	232	−24	(−113)	+6 (+13)
lactate .	.013	—	140	467	439	274	304	−6	(−28)	+11 (+30)
lactate	.013	—	105	358	337	193	232	−6	(−21)	+20 (+39)
lactate .	.013	—	140	467	439	274	304	−6	(−28)	+11 (+30)
lactate .	.013	—	120	360	360	191	222	0	(0)	+16 (+31)
lactate .	.013	+	120	358	355	215	255	0	(−3)	+19 (+40)
lactate ...	.013	—	100	308	302	221	246	0	(−6)	+11 (+25)
lactato ..	.013	+	100	318	307	220	252	−3	(−11)	+15 (+32)
lactate	.013	—	90	288	263	155	237	−9	(−25)	+53 (+82)
lactate	.013	+	90	276	288	163	261	+4	(+12)	+60 (+98)
lactate	.013	—	90	361	358	181	207	0	(−3)	+14 (+26)
lactate	013	+	90	332	357	184	251	+8	(+25)	+36 (+67)

* Figures in parentheses represent the differences in O₂ consumption in mm³ produced by ascorbic acid.

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than that of the control sample. On statistical analysis it was found that the average increase in O_2 consumption (expressed in mm³) produced by ascorbic acid on narcotized brain was significantly higher than that produced on normal

TABLE 3

The effect of ascorbic acid on aerobic glycolysis and oxidation of glucose by brain narcotized by pentobarbital

DURATION OF EXPERIMENT	ADDITION	GLUCOSE REMOVED	LACTATE FORMED	GLUCOSE OXIDIZED (BY DIFFERENCE)	EFFECT OF ASCORBIC ACID ON GLUCOSE OXIDIZED (EXPRESSED AS PER CENT)
min.		mgm.	mgm.	mgm.	
90	no addition	1.12	.25	.87	
	ascorbic acid	1.19	.30	.89	0
	pentobarbital	1.43	.88	.55	
	pentobarbital + ascorbic acid	1.35	.58	.77	+40
120	no addition	.93	.25	.68	
	ascorbic acid	1.19	.21	.98	+44
	pentobarbital	1.25	.78	.47	
	pentobarbital + ascorbic acid	1.22	.26	.95	+100

The concentration of glucose in these experiments was .0133 M.

TABLE 4

The effect of ascorbic acid on lactic acid oxidation by normal and narcotized brain

DURATION OF EXPERIMENT	ADDITIONS	LACTIC ACID REMOVED mgm.		EFFECT OF ASCORBIC ACID ON LACTIC ACID REMOVED (EXPRESSED AS PER CENT)
		Without ascorbic acid	With ascorbic acid	
min.				
140	no addition	.50	.51	+2
	pentobarbital	.26	.29	+12
120	no addition	.50	.53	+6
	pentobarbital	.41	.50	+22
90	no addition	.39	.32	−18
	pentobarbital	.19	.26	+37
90	no addition	.40	.41	+2
	pentobarbital	.21	.30	+43

The concentration of dl lactic acid in these experiments was 0.0133 M.

brain. Although there is some unexplained variation in the results of experiments presented, they are consistent in showing that ascorbic acid reduces the inhibition, produced by pentobarbital, of oxidation by brain of glucose, lactate and pyruvate, thus tending to return the metabolism to that of the control tissue.

Cytochrome c appeared to enhance the effect of ascorbic acid in some cases but the effect was variable (table 2).

In order to demonstrate that the effect of ascorbic acid was actually due to an increased oxidation of the substrate analyses for remaining substrates were done in several experiments in which glucose and lactate were substrates. The results presented in tables 3 and 4 show that the effect was truly one of increased oxidation of substrate.

In the presence of pentobarbital the anaerobic phase of carbohydrate metabolism, namely the breakdown of glucose to lactic acid, is increased. That is, pentobarbital increases the aerobic glycolysis of brain. When ascorbic acid is added to brain the glycolysis is decreased (table 3) and the oxidation is increased, as indicated by the increase in oxygen consumption (table 2) and increased removal of glucose, not accounted for by lactic acid formation (table 3). Results of analysis for lactic acid, in experiments in which this was used as a substrate agree with those obtained from measurements of oxygen consumption in showing that the addition of ascorbic acid leads to an increased oxidation of this metabolite by brain when narcotized by pentobarbital (table 4).

DISCUSSION. The results show that ascorbic acid can reduce the inhibitory effect of pentobarbital in brain oxidations by increasing the metabolism of the added substrate. Since in control experiments without pentobarbital, ascorbic acid does not increase the oxidation consistently, it would appear that normal metabolism is not greatly affected. However, when pentobarbital is present ascorbic acid does produce a considerable increase in metabolism. This indicates that ascorbic acid, with a redox potential which is higher than that of flavoprotein, may offer an alternate pathway for the transport of hydrogen when normal metabolism is blocked. This may be pictured as follows:
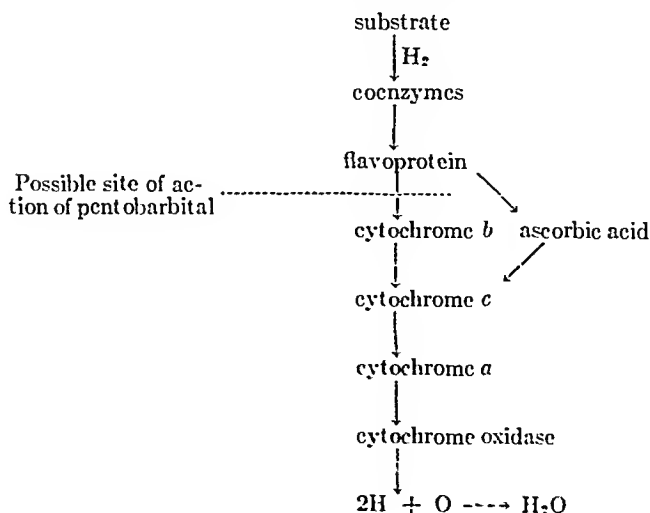
Kellie and Silva (15) found that ascorbic acid can produce similar effects in liver poisoned with fluoride, arsenite, maleate, etc.

Since cytochrome c is essential for the enzymatic oxidation of ascorbic acid (16, 17) by cytochrome oxidase the effect of increasing the concentration of cytochrome c was investigated. It was found that pentobarbital-inhibition can be further reduced when cytochrome c is added to the medium with the ascorbic acid. This action of cytochrome c probably signifies that the initial concentration of cytochrome c in the tissues was not optimal. Adding cytochrome c alone to brain tissue inhibited by pentobarbital has no effect, since the block produced by pentobarbital is above cytochrome c in the metabolic path. When added with ascorbic acid, however, it may increase the rate of oxidation of ascorbic acid to dehydroascorbic acid which may then accept hydrogen and again become reduced.

Several authors have already shown that in experiments *in vivo*, ascorbic acid is antagonistic to barbiturates. Green and Musulin (6) found a correlation between the ascorbic acid level and the response produced by phenobarbital and pentobarbital in guinea pigs, the higher the vitamin level the less the depression produced by the barbiturates. Richards, Kueter and Klatt (7) report that guinea pigs on a diet low in ascorbic acid slept longer after a given dose of pentobarbital than the same animals when on a normal diet Einhauser (8) found that ascorbic acid enhanced the antagonism of adrenal cortical extract to barbiturates administered to rats.

Since ascorbic acid has been found to be antagonistic to pentobarbital both *in vitro* and *in vivo*, it is possible that the mechanism of action is the same in both cases. In the experiments reported in this investigation, ascorbic acid appeared to reduce the inhibition of metabolism produced by pentobarbital by offering an alternate route for the oxidation of substrate, thus by-passing the block produced by pentobarbital. If this be the explanation for the antagonism of ascorbic acid to pentobarbital *in vivo* also, it would add support to the theory that the production of anesthesia is intimately connected with the inhibition of metabolic processes in brain produced by anesthetics.

SUMMARY

1. Ascorbic acid can reduce the inhibition produced by pentobarbital in the oxidation of carbohydrate by rat brain *in vitro*.

2. It also reduces the aerobic glycolysis of brain produced by pentobarbital when glucose is substrate.

3. Cytochrome c increases the effect of ascorbic acid in some cases by further reducing the inhibition produced by pentobarbital.

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THE COMPARATIVE PHARMACOLOGICAL ACTIVITY OF BETA-CYCLOPENTYLETHYLAMINE AND BETA-CYCLOPENTYLISOPROPYLAMINE

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Since the fundamental study by Barger and Dale (1) of the relationship between the chemical constitution and physiological action of the sympathomimetic amines, numerous investigations have been made of many structurally and pharmacologically related compounds. A few publications have described the pharmacology of compounds in which cyclic arrangements other than the benzene ring have been incorporated into the general sympathomimetic structural pattern (2–5). We have investigated two new compounds of this general type, beta-cyclopentylethylamine and beta-cyclopentylisopropylamine, and compared them with the spatially related compounds, beta-phenethylamine and amphetamine (beta-phenisopropylamine).[1]

$CH_2CH_2NH_2$ $CH_2CH—CH_3$ $CH_2CH_2NH_2$ $CH_2CH—CH_3$
　　　　　　　　　　　　NH_2　　　　　　　　　　　　　　NH_2

Cyclopentylethylamine　　　　　　　　Phenethylamine

　　　Cyclopentylisopropylamine　　　　　　　　　　Amphetamine

Experiments herein reported involve the assaying of these agents for pharmacological activity by the commonly employed procedures.

BLOOD PRESSURE EFFECTS. *1. Method.* Crude preliminary experiments on barbitalized dogs indicated that repeated injections of cyclopentylisopropylamine give diminished effects as do phenisopropylamine (6) and ephedrine (7) and that cross tachyphylaxis between the compounds under investigation can occur (fig. 1A). Figures 1B and 1E illustrate unsuccessful attempts to use the procedure suggested by Alles (8) to minimize these tachyphylactic effects in serial injections by using minimally effective doses at prolonged time intervals. However, it was found that dose-effect curves (9) for cyclopentylethylamine and phenethylamine could be obtained for comparison with similar epinephrine response curves, since these two agents did not apparently develop tachyphylaxis under the conditions used (fig. 1C and 1D). In order to avoid the error that can be introduced by tachyphylaxis we resorted to a modification of the method of Chen (7) of standardization

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[1] In accordance with established usage, these compounds are called "phenethylamine" or beta-phenethylamine, and amphetamine or phenisopropylamine, instead of the systematic chemical names 2-phenyl-ethylamine and 1-phenyl-2-propylamine.

Fig. 1. Dogs. 330 mgm. of Na Barbital per kgm. Blood pressure in mm. Hg, ordinate. Doses of drugs, in micrograms/kgm., abscissa. Time interval between injections, 6–8 minutes, unless otherwise indicated. EPI = l-epinephrine (as hydrochloride), C532 = cyclopentylisopropylamine sulfate, PE = beta-phenethylamine sulfate, AM = amphetamine sulfate, and C522 = cyclopentylethylamino sulfate.

with an agent, such as epinephrine, that neither produces nor is affected by this phenomenon, and then injecting a test dose of the agent under consideration.

Dogs. Forty-eight apparently healthy, adult mongrel dogs (5–16 kgm.) were used; these were anesthetized with sodium barbital (330 mgm./kgm.) administered intraperitoneally 90 minutes prior to use. Blood pressure usually was recorded by an ink-writing mercury manometer connected to a cannula in the carotid artery. Seven soot kymograph records were prepared for illustrative purposes. Six dogs were cannulated with a modified Hamilton manometer (10). Twenty-eight of the dogs were vagotomized and the remainder received atropine sulfate (1 mgm./kgm.) intravenously. The animals were standardized with graded doses of epinephrine (1–12 μgm./kgm.) and the test dose of the agent, as a 1 per cent solution of the sulfate salt, was injected into the femoral vein.

Cats. Since much of the earlier work with sympathomimetic amines has been done with the cat, we prepared 20 cats in the same manner as the dogs except that the median circumflex branch of the femoral artery was stubcannulated and the pressure changes recorded with a modified Hamilton manometer. The customary standardization with epinephrine was performed and the agent under investigation was injected every 15 minutes for 5 injections. Portions of typical records are reproduced in fig. 2.

Rabbits. Githens (11) has indicated that rabbits are more sensitive than cats or dogs to epinephrine, and it has been reported that phenethylamine often produces depressor responses in this animal (12, 13). Sixteen rabbits were prepared in the same manner as the cats, except that only 270 mgm. of sodium barbital per kgm. was used as anesthetic. It was found that it was unnecessary to give over 4 μgm. of epinephrine per kgm. during the standardization procedure.

2. Results. The results of the epinephrine-agent equivalence are summarized in the table. The use of a fixed dose of 1 mgm. of agent per kgm. is comparable to the work of others in the field, and the data obtained with phenethylamine and phenisopropylamine are comparable to those obtained by other workers using similar procedures (6, 14, 15). To comply with the dictum of Barger and Dale (1) that sympathomimetic agents should be compared on equimolecular bases, the data available have been expressed in terms of moles of amine base contained in one milligram of amine sulfate and compared with the pressor equivalent number of moles of epinephrine base. The activity ratios are given in the table.

These agents produce more slowly developing and more prolonged rises in blood pressure than does epinephrine. The duration of effect of the cyclopentylisopropylamine and phenisopropylamine is 3 to 10 times as great as the corresponding ethylamines. The heart rate increases, cardiac output apparently increases (16), and peripheral vasoconstriction occurs (17). Repeated administration of the substituted isopropylamines leads to the development of a characteristic fall in blood pressure (see figure 2C) due to peripheral vasodilation, as evidenced by the increased rate of diastolic run-off and by disappearance of the typical standing waves (17, 18).

The change in relative activity of these compounds from the dog to the cat can be attributed easily to species difference. However, the fact that the change is an increase in potency in three compounds and a decrease with the other, in relation to epinephrine, may indicate that a more fundamental relationship between constitution and action exists in the cat.

The pressure response to these compounds in the rabbit is quite complex.
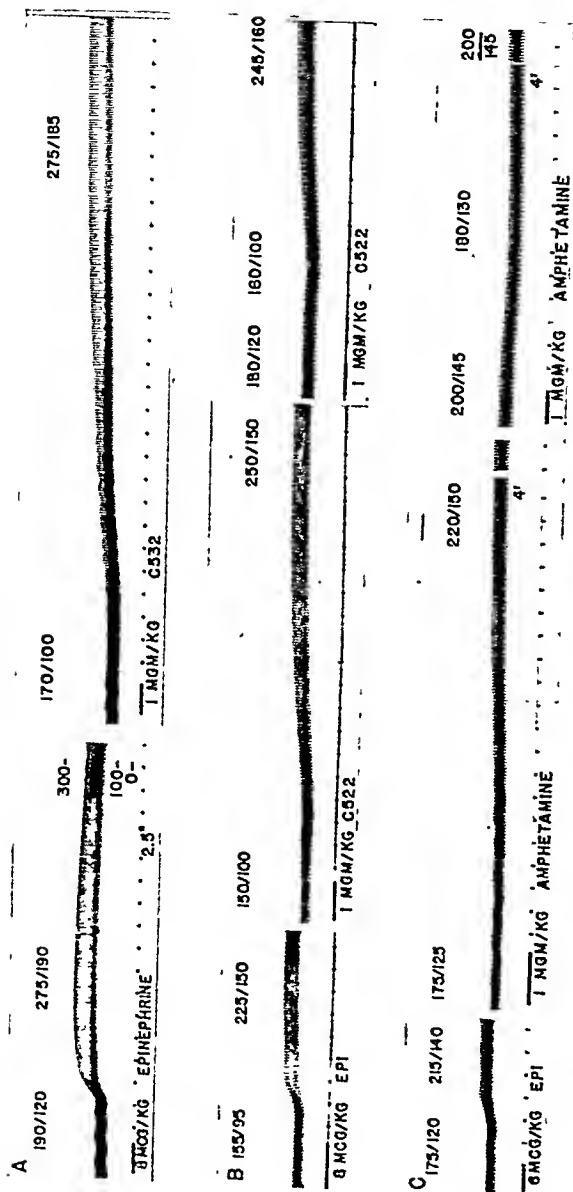
FIG. 2. Cats. 330 mgm. of Na Barbital per kgm. Optical manometer records. Calibration in mm. Hg. Cat A received 8 micrograms of l-epinephrine per kgm. micrograms of l-epinephrine per kgm. and 8′ later 1 mgm. of cyclopentylisopropylamine sulfate per kgm. Cat B received 8 micrograms of l-epinephrine per kgm. and 8′ later 1 mgm. of cyclopentylisopropylamine sulfate per kgm. Three injections of this drug were given at 15-minute intervals (not shown) and the final injection is reproduced. Cat C received 6 micrograms of l-epinephrine and 7′ later 1 mgm. of amphetamine sulfate per kgm. and 15′ later an additional 1 mgm. of amphetamine sulfate per kgm.
Time line = 2.5 second intervals. Duration signal for nil injections.

There is an initial vasodilation and perhaps myocardial weakening followed by peripheral vasoconstriction with increased pulse pressure. Repeated injections of these compounds leads to decreased pressor response and often a decrease in the primary depressor response. These agents are far less potent in relation to epinephrine in the rabbit than they are in the dog and cat.

PERFUSED RABBIT HEART. Since these agents produce cardioacceleration and perhaps increased cardiac output, rabbit hearts were prepared for perfusion by the method of Langendorf and rate, force of contraction, and coronary outflow were recorded. Test doses of epinephrine and the agents under investigation were added alternately to the perfusion fluid. In every instance, regardless of the amount of agent added to the perfusion fluid, only decrease in rate, in force of contraction, and in amount of coronary perfusate were obtained with phenethylamine, amphetamine, cyclopentylethylamine and cyclopentylisopropylamine. Following small amounts of these agents (final concentration 1:1000,000–1:10,000), epinephrine had its usual effects, but larger amounts

TABLE 1

COMPOUND	AVERAGE NUMBER OF MICROGRAMS OF EPINEPHRINE EQUIVALENT IN PRESSOR EFFECT TO ONE MGM OF AMINE SULFATE			AVERAGE NUMBER OF MOLECULES OF AMINE EQUIVALENT IN PRESSOR EFFECT TO ONE MOLECULE OF 1-EPINEPHRINE	
	Dog	Cat	Rabbit	Dog	Cat
Cyclopentylethylamine	4.04 (6)*	7.83 (5)	1.0 (4)	320	173
Phenethylamine	10 30 (13)	8 11 (5)	2.6 (4)	125	159
Cyclopentylisopropylamine	5 12 (6)	7.96 (5)	1.7 (4)	243	157
Amphetamine	4.16 (23)	5 94 (5)	1.1 (4)	288	201

* The number of animals used is expressed parenthetically.

(1:2,000–1:1,000) apparently damaged the myocardium so severely that the epinephrine response was diminished. Barbour and Frankel (19) and Alles and Knoefel (12) found similar effects with phenethylamine.

ACTION ON BRONCHI. None of these agents gave evidence of bronchodilation in histamine-constricted guinea pig lungs prepared by the method of Tainter, Pedden, and James (20), although good bronchodilation by epinephrine was obtained. Apparently the simple sympathomimetic agents closely related to phenethylamine are unable to produce effective bronchodilation or antagonism of histamine.

ACTION ON ISOLATED RABBIT JEJUNUM. Sections of jejunum from 5 rabbits were placed in oxygenated Ringer-Locke solution. Concentrations of less than 1×10^{-3} molar (about 160–180 μgm./ml.) of phenethylamine, amphetamine, cyclopentylethylamine, and cyclopentylisopropylamine had little stimulant effect or no action in all segments. 1×10^{-3} molar phenethylamine had a contractile action on strips of 2 rabbits, and a concentration of 2×10^{-3} molar of the four agents produced approximately fifty per cent relaxation or diminution of tone and amplitude of contraction in all segments. The agents were virtually indistinguishable in action at this concentration.

MYDRIATIC ACTION. Two tenths ml. of a 2 per cent solution of any of these four agents instilled into the intact cat or rabbit eye caused definite mydriasis within 20 minutes. Phenethylamine was placed in the left eye and cyclopentylethylamine was placed in the right eye of one subject; amphetamine was placed in the left eye and cyclopentylisopropylamine was placed in the right eye of another subject. Maximal dilation was obtained within 30 to 45 minutes and some mydriasis was evident for as long as 2 hours. Less concentrated solutions of the substituted ethylamines had little or no action. Chen, Wu, and Henriksen (21) found no mydriatic action in the rabbit eye with phenethylamine in a concentration of M/20 (about 0.8 per cent).

ACTION IN MAN. *1. Action on nasal mucous membrane.* Using one per cent solutions of the related agents, 1 ml. of each was placed on similar sized cotton pledgets and placed in the right and left nostril of an allergic individual. The subject was unable to differentiate between the various agents applied on 4 successive days. Chen, Wu, and Henriksen (21) found one per cent solutions of phenethylamine produce nasal mucous membrane constriction, as do ephedrine and similar agents.

2. Oral administration in man. A young adult male weighing 76 kgm. received 50 mgm. of cyclopentylisopropylamine sulfate two and one half hours after a light meal. The agent was taken orally with 250 ml. of warm water. The systolic and diastolic blood pressure and the pulse rate were recorded every 15 minutes for 3 hours while the subject remained sitting quietly. There was no significant change from the normal 120/78 blood pressure and pulse rate of 72. There were no signs of restlessness or talkativeness. The patient did not complain of untoward symptoms. Twenty milligrams of amphetamine sulfate taken orally by this individual usually produce a rise of 15 mm. of Hg. in the systolic blood pressure, with some slowing of the pulse, increased talkativeness, restlessness, and some difficulty in going to sleep at night. Apparently the substitution of the cyclopentyl group for the benzene ring in amphetamine eliminates the characteristic stimulant actions for man.

Fifty milligrams of phenethylamine sulfate and a similar dose of cyclopentylethylamine sulfate had no demonstrable effect when given at one-week intervals. Chen, Wu, and Henriksen (21) found no action in man with oral doses of 50 mgm. of phenethylamine, although Chen (22) found a slight lowering of blood pressure with doses of 150 mgm.

DISCUSSION. In a qualitative way, the results are entirely in accord with the biochemorphological speculation that the cyclopentane ring can substitute for the benzene ring in such simple sympathomimetic amines as these. The quantitative relationships are not readily explainable. With local organs and isolated tissue segments, where the various factors of absorption, excretion, and relative partitive transportation are minimized or play no appreciable role, these agents are all of very similar quantitative activity. In the case of their action on the many structures that influence the overall pressure relationships in the cardiovascular system, the various molecular size and physical property differences are apparently able to influence the activity of the agents. Barger and Dale questioned the concept of various types of effector cells or organs and felt

There is an initial vasodilation and perhaps myocardial weakening followed by peripheral vasoconstriction with increased pulse pressure. Repeated injections of these compounds leads to decreased pressor response and often a decrease in the primary depressor response. These agents are far less potent in relation to epinephrine in the rabbit than they are in the dog and cat.

PERFUSED RABBIT HEART. Since these agents produce cardioacceleration and perhaps increased cardiac output, rabbit hearts were prepared for perfusion by the method of Langendorf and rate, force of contraction, and coronary outflow were recorded. Test doses of epinephrine and the agents under investigation were added alternately to the perfusion fluid. In every instance, regardless of the amount of agent added to the perfusion fluid, only decrease in rate, in force of contraction, and in amount of coronary perfusate were obtained with phenethylamine, amphetamine, cyclopentylethylamine and cyclopentylisopropylamine. Following small amounts of these agents (final concentration 1:1000,000–1:10,000), epinephrine had its usual effects, but larger amounts

TABLE 1

COMPOUND	AVERAGE NUMBER OF MICROGRAMS OF EPINEPHRINE EQUIVALENT IN PRESSOR EFFECT TO ONE MGM OF AMINE SULFATE			AVERAGE NUMBER OF MOLECULES OF AMINE EQUIVALENT IN PRESSOR EFFECT TO ONE MOLECULE OF l-EPINEPHRINE	
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THE ROLE OF THE LIVER IN THE DETOXICATION OF THIOPENTAL (PENTOTHAL) AND TWO OTHER THIOBARBITURATES*

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Prior to 1940 it was assumed that thiopental (Pentothal) was degraded primarily by the liver (1, 2, 3). In that year, Scheifley and Higgins (4) reported that partial hepatectomy in rats did not increase the duration of action of this thiobarbiturate. The following year, Richards and Appel (5) likewise concluded that this drug was not destroyed by the liver, on the basis of experiments in which the duration of action of Pentothal was not prolonged in rats previously treated with carbon tetrachloride. Masson and Beland (6) in 1945, using partially hepatectomized rats, reached the same conclusion as Scheifley and Higgins in regard to the liver as the major site of the detoxication of Pentothal. More recently Richards (7) presented evidence that this drug is destroyed by heparinized human or rabbit blood and states, "experimental evidence from this laboratory and other investigators definitely indicates that the liver is not of outstanding importance in the inactivation of this drug "

In view of the lack of harmony between these findings and earlier results implicating the liver as the primary detoxicating organ for the shorter-acting barbiturates, it was felt that a thorough investigation of the role of the liver in the detoxication of Pentothal was merited. In addition, the possibility of the detoxication by the liver of two other thiobarbiturates, 5-allyl-5-(1-methylbutyl)-2-thiobarbituric acid and 5-ethyl-5-isoamyl-2-thiobarbituric acid (Thioethamyl), was investigated

METHOD Four different technics (three in vivo and one in vitro) were used to determine whether or not the liver degrades Pentothal and the two other thiobarbiturates

I *Production of liver dysfunction by the use of a hepatotoxic agent.* In each experiment, albino mice, chosen without regard to sex, were divided into two groups (A and B). The weight range and mean weight of each group were approximately the same Each animal was then injected with the thiobarbiturate being tested and the duration of action was determined as the time elapsing between loss and return of the righting reflex. Three days later, all mice in the B groups were given carbon tetrachloride in peanut oil (table 1), and 24 hours later, all animals (both A and B groups) were reinjected with the same drug at the same dosage level as had been used previously The mean difference in duration of action between the first and second injection was calculated for each group and the data analyzed statistically

II *Reduction of the amount of functioning liver tissue by subtotal hepatectomy.* Twenty-two rats, weighing 150-200 grams, were injected intravenously with Pentothal Sodium (table 2), and the durations of action determined as the length of time expiring between loss and return of the righting reflex Six days later, 10 of these animals (Group 2) were subtotally hepatectomized by a modification of the method of Selye, Stehle and Collip (8) At this

that the only difference would be in the sensitivity of these effectors towards various agents. From the available data one is unable to delineate the mechanism of action of these agents, but the concept of Burn (23) that agents of the ephedrine and tyramine type act by stimulating sympathetic nerve endings (not the myoneural junction where epinephrine acts) appears very attractive. That epinephrine has a similar response before and after these agents have been effective, and even after their effectiveness has disappeared, leads one to look skeptically upon the concept that these agents act by inhibiting the enzymes that destroy or inactivate epinephrine or naturally occurring sympathin.

SUMMARY

The pharmacological activity of cyclopentylethylamine and cyclopentylisopropylamine has been compared with beta-phenylethylamine and amphetamine. The pressor activity of these compounds in barbitalized dogs, cats, and rabbits has been determined and compared with epinephrine. The action of these agents in the isolated guinea pig lung, on isolated rabbit heart, rabbit jejunum, in the intact rabbit and cat eye, on the nasal mucous membrane of man, and in man following oral administration has been determined.

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and the heat-inactivated liver blotted with a filter paper brush. Next an equal amount (30–50 cc., depending upon the amount of liver present) of the Pentothal solution in buffer was pipetted into each flask. These were shaken throughout the incubation period in a water bath at 38°C. with a stream of oxygen circulating above the solutions.

After incubation, both flasks were heated in boiling water for three minutes and each solution was then decanted into a separatory funnel. Thirty cc. of 1 N HCl was added to the liver remaining in each flask, agitated and decanted into the separatory funnel, followed by two 50 cc. portions and one 25 cc. portion of ether. The mixture in the separatory funnel was shaken one minute and the ether layer decanted into a flask for evaporation. Two successive extractions with 100 cc. of ether and two with 75 cc. were carried out in the same manner. The pooled ether layers from all extractions were then evaporated under a stream of air and the residue washed with petroleum ether until a dry, white, crystalline product was obtained. The two samples were then cooled and 15 cc. of ether added to each. A one cc. aliquot of each was quickly transferred to colorimeter tubes and evaporated to dryness. The Pentothal in the two samples was then chemically estimated by the colorimetric method of Raventós (11). Preliminary experiments were carried out, utilizing the method described, to recover known amounts of Pentothal from aqueous solutions containing liver. Recoveries ranged from 90 to 102 per cent.

The ether remaining in the flasks was again evaporated to dryness and the Pentothal present was alkalinized and made up to what would have been a 0.5% solution (on the basis of the amount originally present) if none had been destroyed. This solution was used for biological assay. For this assay, twenty mice of approximately the same weight (± one gram) were chosen. Ten mice received 0.009 cc. per gram intravenously of the above Pentothal solution from heat inactivated liver. The other ten mice were given the same dose in cc. of the Pentothal solution which had been incubated with surviving liver. Mean durations of action were determined for the two groups and the results analyzed statistically.

The solution remaining after biological assay was reextracted with ether, the residue sublimed, and the melting point of the sublimate determined.

Experiments 5 and 6 were carried out with less than 0.5 gm. of liver nunce, utilizing the traditional Warburg technic for studying tissue respiration. In each experiment six Warburg flasks were used: two contained 4 cc. of the Pentothal-buffer-glucose solution plus heat inactivated liver, two contained 4 cc. of the same Pentothal solution plus respiring liver; and two contained the buffer solution without Pentothal plus respiring liver. After ten minutes of oxygenation and equilibration, manometer readings were made at fifteen minute intervals for four hours. Oxygen uptake was calculated. At the end of the incubation period the KOH in the center well was removed by blotting with filter paper, 5 cc. of distilled water was added to each flask and they were then heated at 93°C. for three minutes. Preliminary experiments had shown that there was no appreciable breakdown of Pentothal by this method. The solution in each flask was decanted through gauze in the stem of a small funnel into a glass stoppered 30 cc. centrifuge tube. Two cc. of 1 N HCl was added to each flask and agitated with the remaining liver. This was then decanted off into the centrifuge tube and three 5 cc. portions of ether used to wash out each flask. The ether was added through the gauze to the previous material and the tubes shaken vigorously for one minute. The ether layer was decanted into a colorimeter tube and two additional extractions of the aqueous phase were carried out with 10 cc. portions and two with 5 cc. portions of ether. The pooled ether layers in the colorimeter tubes were evaporated to dryness, and small amounts of petroleum ether were added several times and evaporated until a dry product was obtained. The material in the tubes was then estimated colorimetrically.

RESULTS. I. There was no significant alteration of the duration of action of Pentothal administered intravenously to normal mice in a dose of 58 mgm. per kgm. at a four day interval (table 1). However, when carbon tetrachloride (1 cc./kgm.) was given orally 24 hours prior to injection of this thiobarbiturate,

time, 85 to 90 per cent of the liver was removed. Twenty-four hours later, all rats were reinjected with the same dose of Pentothal Sodium as had been used previously. Mean difference in duration of action between the two injections was determined for each group and the results statistically analyzed.

III. *Reduction of blood flow through the liver by production of an Eck fistula.* A large series of rats, weighing 150–200 grams, were operated at various times and an Eck fistula produced by a modification of Whitaker's method (9). At least two weeks postoperatively, various groups in this series were injected with different barbiturates and the mean duration of action in each case was compared with that obtained using the same dose of barbiturate in a group of unoperated controls of the same weight. To test the legitimacy of this method in significantly reducing blood flow through the liver and hence prolonging the action of a compound known to be primarily detoxified in the liver, pentobarbital sodium was injected intravenously into 15 unoperated controls and 16 operated animals (table 3). To determine whether or not the operation altered the duration of action of a compound primarily excreted by the kidneys and not detoxified by the liver, sodium barbital was used. This

TABLE 1

Effect of liver damage on the duration of action of certain thiobarbiturates in mice

DRUG	GROUP	NUMBER OF ANIMALS	MEAN DURATION OF ACTION—MIN.		MEAN DIFFERENCE IN DURATION OF ACTION		*t* VALUE OF DIFFERENCE
			1st Injection	2nd Injection (4 days after 1st.)	Min.	Per cent	
Pentothal Sodium 58 mg./ kg. 2% Soln. I.V.	A	22	12.3	13.5	+1.2	+9.7	0.797
	B*	21	14.5	184.0	+169.5	+1169.0	3.41
5-allyl-5-(1-methylbutyl)- 2-thiobarbituric acid 45 mg./kg., 1% Soln. I.V.	A	25	22.4	25.6	+3.2	+14.3	1.77
	B*	29	19.2	149.2	+130.0	+676.0	4.52
Thioethamyl 80 mg./kg., 2% Soln. I.V.	A	24	10.6	7.2	−3.4	−32.1	3.03
	B*	23	9.5	234.1	+224.6	+2364.0	2.75

* Each mouse in this group received 0.02 cc./gm. of a 20% solution of carbon tetrachloride in peanut oil 24 hours before the second injection.

drug was administered intravenously to a group of 31 rats, 15 unoperated and 16 operated, (table 3). Following these preliminary experiments, the thiobarbiturates listed in Table 4 were administered intravenously, in the dose indicated, to unoperated rats and rats with Eck fistulae. The mean duration of action of the drug in each group was obtained and all data analyzed statistically.

IV. *Destruction in vitro by rat liver tissue.* An 0.08% solution of Pentothal in calcium-free Ringer-M/60 phosphate buffer was prepared by adding sufficient alkali to convert the acid into the water-soluble sodium salt plus one part in sixty of M/4 Na_2HPO_4 to provide additional buffering capacity. The pH of the final solution was adjusted to 8.05 with 1 N HCl, and sufficient glucose was added to make a 0.2% solution.

The liver used in these experiments was obtained from rats after decapitation. The tissue was sliced free-hand or minced in a Seevers-Shideman mincer (10). In the first four experiments (table 5), an equal weight of prepared liver was placed in two 125 cc. Erlenmeyer flasks. After adding a small amount of distilled water to one flask, it was suspended for three minutes in a boiling water bath. The water was then decanted from the tissue

and the heat-inactivated liver blotted with a filter paper brush. Next an equal amount (30–50 cc., depending upon the amount of liver present) of the Pentothal solution in huffer was pipetted into each flask. These were shaken throughout the incubation period in a water hath at 38°C. with a stream of oxygen circulating ahove the solutions.

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the mean duration of action in 21 mice was significantly increased 1169 per cent. The second injection of 5-allyl-5-(1-methylbutyl)-2-thiobarbituric acid (45 mgm./kgm.) in control mice produced a mean increase in duration of action of 14.3 per cent which was not statistically significant. In the mice which received carbon tetrachloride prior to the second administration of this thiobarbiturate, its mean duration of effect was increased significantly 676 per cent. In contrast to the above two drugs, the second injection of Thioethamyl produced a significant decrease in duration of action in normal mice. However,

TABLE 2

Effect of subtotal hepatectomy on the duration of action of Pentothal in rats

DRUG	GROUP	NUMBER OF ANIMALS	MEAN DURATION OF ACTION—MIN		DIFFERENCE OF MEANS		t VALUE OF DIFF.
			First Injection	Second Injection (1 week later)	Minutes	Per cent	
Pentothal Sodium 30 mg / kg. 2% Soln. I V.	1	12	34.4	31.0	−3.4	−9.8	0.651
	2*	10	23.8	219.5	+195.7	+822	2.39

* 85 to 90 per cent of the liver removed 24 hours prior to second injection.

TABLE 3

Effect of altering blood flow through the liver on the duration of action of barbital and pentobarbital

DRUG	TYPE OF PORTAL CIRCULATION	NUMBER OF ANIMALS	MEAN DURATION OF ACTION—MIN	DIFFERENCE OF MEANS		t VALUE OF	
				Min.	Per cent	Mean	Difference of Means
Sodium barbital 250 mg./ kg. 10% Soln I V.	Normal	15	321.6	+1.7	+0.53	22.13	0.00136
	Eck	16	323 3			14 13	
Sodium pentobarbital 35 mg./kg. 2% Soln. I.V.	Normal	15	82.3	+80 0	+97.2	6 83	2.76
	Eck	8	162.3			6.17	

previous administration of carbon tetrachloride increased the duration of action (2364 per cent), similar to the results obtained with the other thiobarbiturates.

II. The mean difference in duration of action of two doses of 30 mgm. per kgm. of Pentothal Sodium given one week apart to a group of 12 rats was 9.8 per cent (table 2). Ten rats which were subtotally hepatectomized 24 hours prior to the second injection of the drug showed a significant mean increase in duration of effect of 822 per cent.

III. Porto-caval anastomosis with ligation of the portal vein in rats did not significantly alter the duration of action of barbital (table 3), a barbiturate known to be disposed of primarily by renal elimination. This would also indicate that the surgery in itself has no influence on the length of action of this

series of drugs. On the other hand, pentobarbital has a much longer effect (97.2%) in animals having Eck fistulae than in normal animals. Such data validate the use of this technic as a means of significantly reducing blood flow through the liver and consequently decreasing the rate of degradation of any compound which employs this organ for its detoxication.

Pentothal (30 mgm./kgm.), which had a mean duration of action of 26.4 minutes in 15 normal rats, showed an average length of effect of 224.6 minutes in animals with Eck fistulae, a significant increase of 751 per cent (table 4). 5-allyl-5-(1-methylbutyl)-2-thiobarbituric acid in a dose of 20 mgm./kgm. exhibited a mean duration of action of 7.0 minutes, much shorter than that obtained with the dosage of Pentothal used. However, even with such a short length of action, reduction in amount of hepatic blood flow prolonged it 249

TABLE 4

Effect of altering blood flow through the liver on the duration of action of thiopental (Pentothal) and two other thiobarbiturates

DRUG	TYPE OF PORTAL CIRCULATION	NUMBER OF ANIMALS	MEAN DURA-TION OF ACTION MIN.	DIFFERENCE OF MEANS		t VALUE OF	
				Min.	Per cent	Mean	Difference of Means
Pentothal Sodium, 30 mg./kg. 2% Soln. I.V.	Normal Eck	15 9	26.4 224.6	+198.2	+751	5.33 8.4	7.76
5-allyl-5-(1-methylbutyl)-2-thiobarbituric acid, 20 mg./kg. 1% Soln. I.V.	Normal Eck	15 15	7.0 24.4	+17.4	+249	5.3 4.99	3.32
25 mg./kg., 2% Soln. I.V.	Normal Eck	13 9	35.7 190.1	+154.4	+432	6.18 3.92	3.16
Thioethamyl, 50 mg./kg. 2% Soln. I.V.	Normal Eck	21 17	27.3 111.7	+84.4	+309	7.89 4.36	3.25

per cent. When the dose was increased so that the mean duration of action in normal rats was in the same range as that obtained with 30 mgm./kgm. of Pentothal, the length of action was increased 432 per cent in animals with Eck fistulae. Similar results were obtained with Thioethamyl, reduction of blood flow through the liver significantly increasing the duration of action of this drug 309 per cent.

IV. Pentothal, incubated with liver slices in vitro for 1 hour (Exp. 1, table 5), underwent a 12.6 per cent breakdown as evidenced by results of the chemical determination. Biological assay of this same material demonstrated that mice, injected with the drug recovered from heat-inactivated liver, exhibited a mean duration of action 14.5 per cent longer than those injected with material incubated with surviving liver. The t value of this difference lay on the border line of statistical significance.

In Experiment 2, chemical determination showed that there was a 16.9 per cent breakdown of Pentothal after incubation with surviving liver for 3 hours. Biological assay disclosed a 37 per cent decrease in mean duration of action of the Pentothal recovered from surviving liver as compared with that incubated with heat-inactivated liver. The t value of the mean duration for the control group, injected with material from heat-inactivated liver, was 6.342 and from surviving liver, 5.754; the t value of the difference between these means was significant (1.925).

In Experiment 3 (slice) and 4 (mince), incubated for 4 hours, chemical determinations showed a 43.3 and 40.1 per cent breakdown, respectively. Mouse assay exhibited 75 and 70 per cent decreases in mean duration respectively,

TABLE 5

The in vitro degradation of thiopental (Pentothal) by rat liver

EXP.	INCUBATION TIME	APPROX. WT. TISSUE	CHEMICAL DETERMINATION			BIOLOGICAL ASSAY		
			Pentothal Recovered—Mg.		Per cent Breakdown	Mean Duration of action—min.		
			Heat inactivated liver	Surviving liver		Heat inactivated liver	Surviving liver	% Diff.
Slices								
	Hours	*Grams*						
1	1	2	28.35	24.75	12.6	7.6	6 5	−14.5
2	3	3	19.5	12.2	16.9	6.9	4.35	−37.0
3	4	6	31.5	17.85	43.3	11.35	2.8	−75.0
Mince								
4	4	5	34.35	20.55	40.1	19.95	5.95	−70.1
5	4	0.468	1.45	0.79	45.5	—	—	—
6	4	0.459	1.38	0.74	46.6	—	—	—

with significant t values of the means and of the differences between the means (Exp. 3, 4.071; Exp. 4, 5.6).

Chemical determinations in Experiments 5 and 6 demonstrated a 45.5 and 46.6 per cent breakdown, respectively, of Pentothal incubated with respiring liver. In these experiments it was found that Pentothal inhibits the oxygen uptake of liver mince from 45 to 52 per cent. Amounts of Pentothal present were too small to permit bioassay.

Results of melting point determinations carried out on recovered material after purification by sublimation showed that Pentothal actually *was* the product recovered and not merely some breakdown product with hypnotic properties which the specificity of the chemical method employed did not exclude.

DISCUSSION. In the experiments reported by Scheifley and Higgins (4), an average duration of action of Pentothal Sodium was obtained on nine rats after intravenous injection of 30 mgm./kgm. Subtotal hepatectomies were then per-

formed in which 70 per cent of the liver tissue was removed, and average durations were again determined at 48 hour intervals for 14 days following surgery. At no time postoperatively did they find an increase in average duration as compared with preoperative values. We account for the very divergent findings reported in this paper with subtotal hepatectomy on the following basis: Higgins and Anderson (12) reported that restoration of the liver following partial hepatectomy in the rat begins toward the end of the first 24 hours, and that the remnant of liver remaining more than doubles itself in 72 hours. In spite of this fact, in planning subsequent experiments, Higgins, in collaboration with Scheifley, allowed 48 hours to elapse before making the first postoperative injection of Pentothal. Thus they removed only 70 per cent of the liver initially and allowed considerable regeneration to occur before proceeding with their experiment. We modified this procedure so that 85–90 per cent of the liver was removed and postoperative injections were made 24 hours following surgery. All animals which did not tolerate such drastic surgery well were eliminated from the series, as well as animals which died subsequently of liver insufficiency, even though they survived and recovered from the Pentothal injection. Considerable evidence is accumulating, especially in cases where Pentothal has been administered to patients with liver damage, that the liver so efficiently detoxifies this drug that very little functioning tissue is required to detoxify the small doses used in man.

Our results could not be reconciled with Scheifley and Higgins also in the fact that we found it impossible, even in normal rats, to inject intravenously 30 mgm./kgm. of Pentothal at less than five day intervals without obtaining increasingly prolonged durations of action and, in some cases, death from respiratory failure following the third or fourth injection. Contrary to this, Scheifley and Higgins apparently observed no such cumulative action of the drug, since their data show at least eight separate injections of Pentothal in rats at two day intervals without an increased duration of action at any time either pre- or post-operatively.

Richards and Appel (5), utilizing carbon tetrachloride liver damage in rats, reported results which they interpreted to mean that the duration of action of Pentothal was not increased 24 hours following administration of the hepatotoxic agent. Their conclusions are based on data from three groups of 10–11 rats in approximately the same weight range: group 1, the controls, exhibited an average duration of action of 44 minutes, following the intravenous injection of 40 mgm./kgm. of Pentothal Sodium; group 2, treated with carbon tetrachloride, demonstrated an average duration of 27 minutes; while group 3, which received the same dose of carbon tetrachloride, gave an average duration of 64 minutes. No statistical analysis of the data was mentioned, so that it would be impossible to determine which, if either, of the means was significant and whether or not the differences were significant. We do not consider this evidence sufficiently conclusive to warrant the conclusion that the authors drew—i.e. "carbon tetrachloride had no effect in prolonging the anesthetic action of this barbiturate."

In the same paper, these authors also presented evidence gained by the use

of a choline-free diet to develop functional liver impairment (fatty infiltration) in rats. They concluded that, "with all three drugs mentioned (barbital, Nembutal and Pentothal) there was a definite, although slight, trend towards occurrence of a longer sleeping time in rats maintained on a choline-free diet." In the case of Pentothal, this conclusion was based on results from one group of rats which showed an increased duration of action and five groups which did not. The fact that barbital also showed this same increase, although known to be excreted by the kidney, would tend to invalidate any definite conclusions which might be drawn from this work. We doubt that sufficient liver damage was produced by this method to cause any definite increase in duration of action, since little evidence exists for the assumption that a mild degree of fatty infiltration could interfere markedly with the ability of the liver to detoxify this drug.

In 1945, Masson and Beland (6), using six animals in a group, found no difference in the duration of action of 30 mgm./kgm. of Pentothal injected intraperitoneally into normal and partially hepatectomized rats 24 hours following surgery. Certain legitimate objections may be made to the methods employed by these workers. First, in rats, there is sufficient individual variation in duration of action of Pentothal following intraperitoneal injection that a significant mean cannot be obtained on only six animals. Second, they have assumed that the absorption of a drug from a severely traumatized peritoneal cavity such as results when partial hepatectomy is performed, is the same as from a normal one. In our repetition of this work, a before and after type of experiment was performed in which the *intravenous* route of administration was employed and, in addition, a sufficient number of animals were included in each group to analyze the results statistically.

It has been reported (13) that patients with liver damage usually tolerate Pentothal as well as any other anesthetic agent but such a conclusion has been based only on clinical impressions. Carefully controlled experiments have not yet appeared in the literature in which the duration of action of a standard dose of Pentothal has been compared in normal individuals and in those with severe liver damage. Such preliminary experiments in this laboratory indicate that the results reported in this paper in regard to the liver as the major organ of detoxication of Pentothal in the mouse and the rat are applicable to the dog and the human. Standard doses of Pentothal have been administered intravenously to humans with marked hepatic dysfunction and to dogs with Eck fistulae. The duration of action of the drug in such instances is significantly longer than in humans with normal liver function or dogs with unaltered portal blood flow.

SUMMARY

1. Carbon tetrachloride liver damage in mice significantly prolonged the duration of effect of Pentothal Sodium and two other thiobarbiturates.

2. Subtotal hepatectomy in rats produced a significant prolongation of the duration of action of Pentothal.

3. Diminished blood flow through the liver, as produced by the Eck fistula

technic in rats, increased the duration of action of Pentothal and the two other thiobarbiturates tested.

4. Pentothal was degraded in vitro by rat liver slices and mince.

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EFFECTS OF CHRONIC DDT INTOXICATION IN RATS ON LIPIDS AND OTHER CONSTITUENTS OF LIVER[1]

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The continued oral administration of DDT (2,2,bis(p-chlorophenyl)1,1,1,-trichloroethane) to animals has been shown by several workers to produce chemical and histological changes in the livers of these animals. Necrotic degeneration (1–3), increase in weight (4) and elevation of the total lipid content (5) of the liver have been reported. It has also been shown that DDT given to dogs accumulates mainly in the body fat (6). On the basis of these findings it was decided to compare the concentrations of several components of the ether-soluble fraction in the livers of normal rats with those in rats chronically intoxicated with DDT, and to measure other liver constituents likely to be affected.

EXPERIMENTAL. *Methods.* In a preliminary series of experiments, only the total lipid[3] concentration in the livers of rats receiving DDT in oil was determined. These animals were part of a group on which tissue metabolic studies have been reported previously (7). Adult rats (male and female) weighing 200–300 gm., received a 2 5 per cent solution of purified DDT (m.p , 108.6–109.5°C.) in corn oil daily by stomach tube for 30 to 100 days. The daily dose was 2 ml. of oil solution, containing 50 mgm. of DDT per kgm of body weight. Control animals received an equivalent amount of oil. All animals had access to a stock diet (Purina Dog Chow) and water at all times and were sacrificed about 18 hours after the last dose. Total lipids were determined on a portion of the liver by the method described for the later experiments.

A more complete study of liver tissue was carried out on female rats (150–180 gm.) which were separated into 3 groups containing 12 animals as controls and 16 in each of the 2 experimental groups. The rats were kept in individual cages and fed *ad lib*. The control group, A, received the stock diet which had been ground in an electrically driven mill. In the same ground chow diet, 0.07 per cent of DDT was incorporated by thoroughly rolling in a ball mill and this diet was fed to the animals of group B. Group C received the ground diet, to which 0.07 per cent DDT and 0.2 per cent choline chloride were similarly added. Two of the rats in group B and 6 in group C died during the course of the experiment; these animals were discarded.

After 36 to 40 days on this regimen each rat was killed by a blow on the head and the liver excised for analysis. Excess blood was rapidly blotted off with filter paper and the whole liver was weighed. A sample was taken for glycogen analysis, which was carried out according to the method of Good, Kramer and Somogyi (8) except that hydrolysis was carried out in 5N H_2SO_4, as suggested by Sjögren (9). A separate portion of about 2 gm. was minced and weighed for lipid analyses. The sample was covered with excess alcohol (about 50 ml.) and allowed to stand for 24 hours at room temperature. The alcohol was then evaporated with the aid of an air stream, the tissue was dried overnight at 105°, and was weighed. The

dried tissue was extracted by adding 50 ml. of ether and the extract was decanted after standing for 24 hours at room temperature. This extraction was repeated, and the ether extracts were combined. The fat-free tissue was then redried and weighed for calculation of total lipids.

Aliquots of the combined ether extracts were used for phospholipid and cholesterol determinations. Cholesterol was determined by a modification of the Liebermann-Burchard reaction. Phospholipid was measured as total phosphorus after digestion and oxidation according to the method of Gortner (10). The phosphorus figures were multiplied by 24 to give average phospholipid values.

RESULTS. The concentration of lipids in the livers of rats which had received DDT in oil for 30 to 100 days was compared with that of control rats which had received only oil and that of comparable normal rats on the stock diet. The results are given in Table 1. The values for the animals which had received only the oil did not differ significantly from those of the normal stock rats. However, there was a significant increase in the liver lipids of the animals which had received DDT.

TABLE 1

Effect of continued administration of DDT in oil on total liver lipids of adult rats

TREATMENT	NO. OF ANIMALS	TOTAL LIPIDS IN DRY LIVER
		Per cent ± s.e.
Stock diet .	10	13.6 ± 0.4
Stock diet + 2 ml corn oil/Kg./day for 30-100 days.	14	14.7 ± 0.8
Stock diet + 50 mg. DDT in 2 ml. corn oil/Kg./day. 30-100 days	13	19.2 ± 0.7

In order to rule out the possibility that the oil used as solvent had affected the changes in lipid concentrations of the rat livers used in the foregoing series, the animals in the second series received DDT incorporated into the regular diet. Excess choline chloride was added to the diet of one of the groups receiving DDT (Group C), so as to determine whether the type of fatty liver caused by DDT administration was in any way related to a choline deficiency or to an increased requirement for choline under these conditions.

The average values for liver lipids, water and glycogen obtained for the control rats (Group A), the rats which received DDT (Group B) and those which received DDT and additional choline (Group C), are shown in Table 2. In all the findings there were no significant differences between the rats which received DDT alone (B) or DDT with choline (C). For purposes of discussion, therefore, Groups B and C are considered together.

The rats receiving DDT gained less weight than did the control animals as is shown in Table 2, in confirmation of similar findings by Laug and Fitzhugh (4). The rats in Groups B and C consumed much less food than did those in Group A. The weight gain by the control Group, A, was low for rats of that age; however, this may have been due to the ground diet or to the summer temperature.

The livers of the animals which had received DDT (B and C) were much larger than those of Group A and correspondingly comprised a much larger percentage of the body weight of the rats (5.7) than did those of the controls (3.7). The per cent of water and of glycogen in the livers were not affected by the administration of DDT. Barron (11) has reported a marked decrease in

TABLE 2

Effects of chronic intoxication of rats with DDT on liver constituents

Group	A	B	C
Addition to Control Diet	None	0 07 per cent DDT	0 07 per cent DDT + 0.2 per cent choline
Number of rats per group	12	14	10
Initial weight of rats—grams	166	163	163
Final weight of rats—grams	188	*172	*168
Final weight of rat livers—grams	6 9	*9 8	*9.6
$\dfrac{\text{Weight of liver} \times 100}{\text{Weight of rats}}$ = per cent	3.7	*5 7	*5 7
$\dfrac{\text{Dry weight of liver} \times 100}{\text{Fresh weight of liver}}$ = per cent	30.2	30.6	30.1
$\dfrac{\text{Glycogen} \times 100}{\text{Fresh weight of liver}}$	3.1 (7)	2.9 (10)	3.0 (6)
Total lipid—mg per liver	306	*554	*528
$\dfrac{\text{Total lipid} \times 100}{\text{Fresh weight of liver}}$ = per cent	4.5	*5.7	*5 5
$\dfrac{\text{Total lipid} \times 100}{\text{Dry weight of liver}}$ = per cent	14.8	*18.5	*18 4
Phospholipid—mg per liver	158	*270	*259
$\dfrac{\text{Phospholipid} \times 100}{\text{Total lipid}}$ = per cent	52.2	49 8	50.4
$\dfrac{\text{Phospholipid} \times 100}{\text{Dry weight of liver}}$ = per cent	7.7	*9 2	*9.1
Cholesterol—mg per liver	23.3	*32.8	*31.3
$\dfrac{\text{Cholesterol} \times 100}{\text{Total lipid}}$ = per cent	7.6	*6.1	*6.0
$\dfrac{\text{Cholesterol} \times 100}{\text{Dry weight of liver}}$ = per cent	1.1	1.1	1.1

* Shows statistically significant difference from control group by t test (12)

Figures in parentheses in the body of the table refer to the number of animals in each group which were used for analysis; all other determinations were carried out on all animals in each group.

the liver glycogen of animals acutely poisoned with DDT. However, this appears to be due to the development of tremors in acutely poisoned animals and to the accompanying increased utilization of carbohydrates Blood glucose and lactic acid levels have been correlated with the severity of the tremors in dogs receiving single doses of DDT.[4]

[4] Sarett, H. P., and Jandorf, B. J., unpublished observations.

The concentrations of *total lipids* in the livers of the rats in Groups B and C were significantly but not markedly greater than in those of Group A. Additional choline in the diet had no effect on the level of fat in the livers of the animals poisoned with DDT. The total lipids, per liver, were increased about 50 per cent over the control figures due to the large increase in weight of the livers and the increase in the per cent of fat.

The concentrations of *phospholipid* (measured as phosphorus) in the livers of the rats were increased in terms of per cent of total dry weight, from 7.7 in the normal animals to 9.2 and 9.1 in the animals receiving DDT alone and DDT with choline, respectively. When the phospholipids are calculated as the percentage of the total lipid, no difference between the three groups is noticeable. On the other hand, the *cholesterol* concentration, in terms of liver dry weight, is the same for all three groups, though cholesterol comprises a significantly lower fraction of the liver lipids in Groups B and C than in Group A. Thus, the phospholipids appear to increase in proportion to the total lipid concentration, whereas cholesterol is increased only in proportion to the weight of the liver.

DISCUSSION. The above results confirm the findings that liver hypertrophy and an increase in fat content accompany the chronic intoxication of rats with DDT. Both of these manifestations may be protective mechanisms. The increase in lipid concentration occurs after administration of DDT either in oil or incorporated into a stock diet.

The phospholipid content of the livers of rats receiving DDT is increased in proportion to the total lipid increase, while the percentage of cholesterol in the total lipids is slightly decreased. The total cholesterol is increased only in proportion to the increased weight of the liver.

The hypertrophied livers of the animals which received DDT contain the same percentage of water and glycogen as do those of the normal animals. The low liver glycogen found in acutely poisoned rats (11) appears to be due only to the induced tremors.

SUMMARY

The chronic intoxication of rats with DDT administered either in oil or mixed in the diet leads to an increased per cent of total lipids in the liver.

With DDT in the diet there is an increase of 40 per cent in the size of the livers but no change in the percentage of water or of glycogen.

The increased total liver lipids are accompanied by a proportional increase in phospholipid content but a smaller increase in total cholesterol. The increase in cholesterol is proportional to the increase in weight of the livers.

The liver lipid changes after administration of DDT are not affected by the inclusion of an additional 0.2 per cent of choline chloride in the diet.

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STUDIES OF THE MECHANISMS OF LIVER AND KIDNEY INJURY

III. METHIONINE PROTECTS AGAINST DAMAGE PRODUCED IN THE RAT BY DIETS CONTAINING PYRIDINE

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The effects of pyridine were investigated because of the possibility that its methylation in the body might lead to a deficiency of substances containing labile methyl groups, with consequent tissue damage. The injury produced in rats by diets containing pyridine, and the modification of the injury by various supplements, have been described in preliminary reports (1, 2), and the morphological aspects of the injury have been discussed in detail elsewhere (3). The present report describes more fully the effects of pyridine on growth, food intake, and survival of the animals, and shows the influence of the protein (casein) level of the diet, and of supplements of methionine, on the results, and the following article of the series (4) considers some of the possible mechanisms involved.

EXPERIMENTAL. *Animals.* Young male rats (Sprague-Dawley, Sherman, and a mixed strain from local colony) after weaning were placed for about a week on stock diet and then transferred to experimental diets.

Diets. The basal diet (diet No. 1[2]) had the following composition:

Casein	10%
Lard.. . . .	20
Sucrose...... ..	30
Corn Starch....	29
Salt Mixture	4
Yeast..	5
Cod Liver Oil....	2

Other experimental diets were prepared by adding various levels of pyridine, casein, and *dl*-methionine to the basal diet. The diets were made up in small quantities and refrigerated in closed containers. Rats were exposed to food for 30 minutes, 3 times a day, and all uneaten food removed from the cages after each feeding.

In later experiments, a diet with refined vitamin supplements, sucrose and hydrogenated vegetable oils in place of the yeast, cod liver oil, starch and lard, and which contained 25% casein and 0.2–0.3% choline chloride (diet No. 3[2]) was employed. Pyridine citrate was substituted for pyridine, and adult rats as well as young rats were used. These animals were exposed to food continuously.

RESULTS. The results with various modifications of the basal diet are shown graphically by the growth curves in figures 1 and 2.

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[2] The basal diet of this report is referred to elsewhere as diet No. 1, where both this diet and diet No. 3 are described in detail (3).

The rats receiving the basal diet alone all survived, and no extensive pathological lesions were observed during the experimental period. The growth rate of the animals on the basal diet was less than that of those on the stock diet.

The addition of 0.1 to 0.2% pyridine to the basal diet caused the animals to stop growing immediately and most of them died within 2 weeks. Examination of the dead animals revealed extensive hepatic and renal injury.

When sufficient casein was added to the pyridine-containing diet to double the casein level, the length and rate of survival of the animals were not markedly
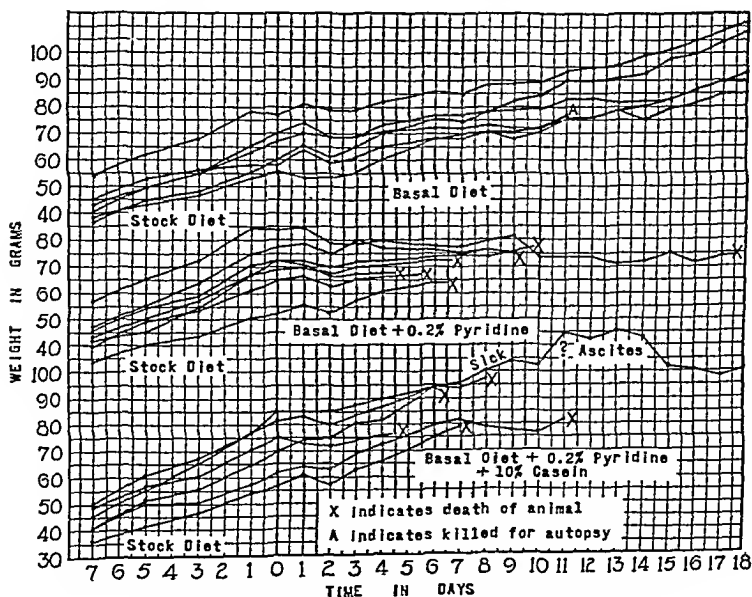
FIG. 1. GROWTH CURVES SHOWING THE EFFECTS OF ADDING PYRIDINE, AND PYRIDINE PLUS CASEIN TO THE BASAL DIET

increased. However, the growth curves differed from those on the lower casein level, in that fairly normal growth continued to the time of death (figure 1).

Addition of small amounts of methionine to the pyridine-containing diet, like raising the casein level in the previous experiment, enabled the animals to grow but did not significantly increase the rate of survival. Larger amounts of methionine, though, caused many of the animals,—from 30 to 90% of the groups in the various experiments that have been done,—to survive throughout the experimental periods of 1 to 2 months (figure 2).

With diet No. 3, 0.7 to 1.0% pyridine citrate was found to produce liver and kidney damage, with death occurring in a large percentage of the animals. The protective effect of 1% additions of methionine to this diet containing 1% pyri-

dine citrate, is demonstrated by the results shown in table 1.[3]   As with the other diets of fairly high casein level, moderately good growth continued to the time of death when the animals were placed on diet No. 3 containing lethal concentrations of pyridine.
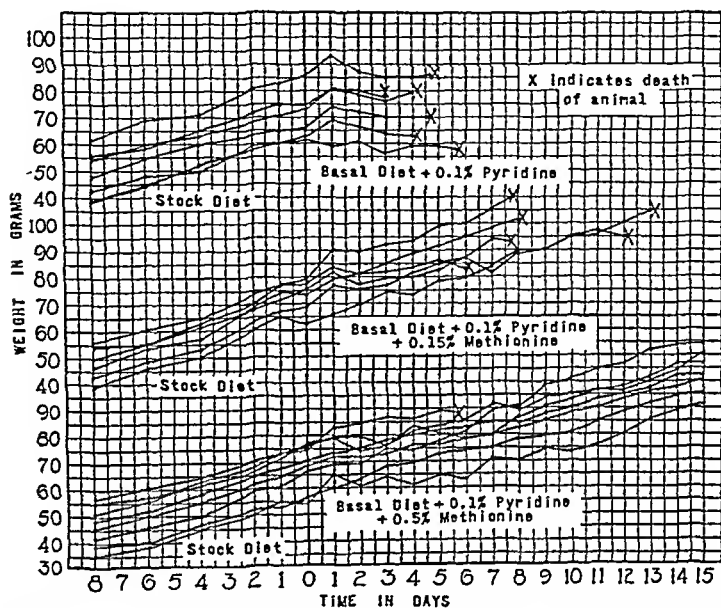
FIG. 2. GROWTH CURVES, SHOWING THE MODIFICATION OF THE PYRIDINE EFFECTS BY VARIOUS CONCENTRATIONS OF METHIONINE, USING THE BASAL DIET

TABLE 1

Results with Diet No. 3

NO. OF ANIMALS*	PYRIDINE CITRATE	METHIONINE	SURVIVING 1 WEEK	SURVIVING 2 WEEKS	SURVIVING 3 WEEKS	SURVIVING 4 WEEKS
	%	%	%	%	%	%
12	1.0	—	75	17	0	0
12	1.0	1.0	83	67	58	42

* Most of these rats weighed approximately 100 grams at the beginning of the experiment.

It is evident from the growth curves (figures 1 and 2), that the food (and pyridine) intake of the animals on the pyridine-containing modifications of the basal

[3] The toxicities of diets containing pyridine have been observed to vary considerably from time to time, apparently with the season of the year, and seem to be least in hot weather. For this reason, comparisons should be made only between groups of animals that were run simultaneously. The data presented in this paper represent high relative toxicities.

diet which also contained methionine, was almost invariably greater than that of the animals on the same combinations without added methionine. Likewise, with diet No. 3 containing pyridine, the average daily food consumption was greater in the groups receiving supplements of methionine than in the controls, but the difference was not as marked as that observed with diets of lower protein level.

DISCUSSION. The problem of differences in food intake of different groups of animals was difficult to control or evaluate. Since the daily food (and pyridine) consumption of the animals receiving supplements of methionine, was as great or greater in most cases than that of similar animals on the same diets without added methionine, it did not seem necessary to do paired-feeding experiments.

Because casein is rather low in its content of sulfur-containing amino acids, and its biological value can be increased (at least in the rat) by the addition of methionine or cystine to the diet, it was conceivable that the protection afforded against the effects of pyridine in the basal diet might have been due to a general metabolic effect of methionine,—that due to making a sub-optimum diet more nearly optimum,—rather than to a specific antagonism of the pyridine effects by methionine. It did not seem very likely, however, that such an action could have outweighed the harmful effects of the increased pyridine intake which occurred on the methionine-containing modifications of the basal diet. In an effort to rule out this "non-specific" action of methionine, diet No. 3, which contained sufficient casein (and choline) to furnish optimum levels of the sulfur-containing amino acids for normal growth (5), was employed, and the results with this diet seemed to indicate that at least a part of the protective effect of methionine was due to that part of the methionine over and above the level required for maximum growth per gram of food under normal conditions.[4] This high protein diet, on the other hand, probably furnished excessive amounts of some amino acids other than methionine and cystine, which had to be metabolized, and an experimental diet of moderate casein level with just sufficient methionine already added to increase the biological value of the diet to the maximum, might have been preferable. Even this combination would have been open to certain objections, and all that can be concluded with certainty is that methionine afforded fairly striking protection under the conditions employed.

CONCLUSIONS

The addition of pyridine to a diet moderately low in protein, produced immediate cessation of growth, and death with liver and kidney injury within 2 weeks in most of the rats receiving the diet.

[4] The possibility that pyridine might have been excreted in combination with cystine or a related substance, thus producing a deficiency of cystine and methionine, or that it might have combined in some manner with the sulphur-containing amino acids of the proteins, rendering these amino acids unusable in the body and causing a "conditioned" deficiency, has been considered. Preliminary experiments have not thus far provided evidence of such a mechanism, and it appeared unlikely, from the observation that growth continued to the time of death in most of the animals which died on the diets of higher protein level or with added methionine, that there was a severe enough deficiency of this type to account for the damage produced by pyridine

When the pyridine-containing diet was supplemented by small amounts of *dl*-methionine (or casein), the animals grew fairly normally but survival was not increased. Larger amounts of methionine produced a considerable increase in length and rate of survival.

Methionine also afforded protection when added to a pyridine-containing diet which already contained sufficient casein (and choline) to furnish optimum levels of the sulfur-containing amino acids for normal growth and development.

Food (and pyridine) consumption was at least as great with, as without, the added methionine.

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STUDIES OF THE MECHANISMS OF LIVER AND KIDNEY INJURY

IV. A Comparison of the Effects of Pyridine and Methyl Pyridinium Chloride in the Rat

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Early investigators regarded liver necrosis and cirrhosis as results of toxic substances acting on the liver, and numerous necrosis-producing agents were found capable, on chronic administration, of producing cirrhosis (1). Many of these substances also produced renal tubular damage, and certain ones have subsequently been found to cause malignant tumors (2, 3). More recently the same pathological manifestations have been observed to develop in animals on diets inadequate in choline and methionine. The production of necrosis and cirrhosis by dietary means has been reviewed by Gyorgy (4), and the production of malignant tumors under similar circumstances was described by Copeland and Salmon (5).

The possibility of a relationship between the processes which follow the feeding of these deficient diets and those which follow the administration of hepatoxic substances was suggested, in the first place, by the similarity of the lesions produced, and, secondly, by the observations that the effects of the toxic substances frequently may be modified by changing the composition of the diet.

In order to investigate the possible relationship of the mechanisms involved in the production of liver and kidney injury by toxic substances, on the one hand, and the causation of similar damage by dietary deficiencies, on the other, the effects of pyridine were investigated (6, 7, 8, 9). His (10) found that when pyridine in the form of the acetate was administered to dogs, methyl pyridinium hydroxide appeared in the urine. Subsequent investigators have observed the methylation of pyridine by several, but not all (11), species of animals. There apparently have been no similar studies reported on the rat. The methylated product has been found in the urine of normal persons (12), arising presumably from coffee and tobacco, and has been reported in the urine of patients with severe burns (13). From the results of other investigations which will be discussed subsequently, it seemed that the methylation of pyridine in the body might be largely at the expense of the methyl groups of choline and methionine, and that it might be possible to produce an intrinsic deficiency of these substances, either generally or at least in the cells where the methylation took place, by the administration of pyridine, and thus produce the same pathological lesions that are caused by diets deficient in choline and methionine. It was realized that there

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is substantial evidence that all of the abnormal effects produced by diets low in choline and the sulfur-containing amino acids may not be due to deficiencies of choline or labile methyl groups alone (14, 15, 16), and that many investigators using diets low in casein as well as choline, have observed a combination of effects which they have sometimes incorrectly attributed entirely to choline deficiency.

When rats were fed diets containing pyridine, acute necrosis and cirrhosis of the liver with calcification and large regenerative tumor-like nodules of the liver, and tubular injury followed by regenerative changes in the kidney were produced (8). These changes were prevented to a considerable extent by the addition to the diet of supplements of methionine (9). These results were compatible with, but did not prove the correctness of, the hypothesis that pyridine might produce injury by a drainage of labile methyl groups.

To further test this hypothesis, it was decided to compare the effects of feeding diets containing stoichiometrically equivalent amounts of pyridine and methyl pyridinium chloride, and to attempt to obtain methyl pyridinium salts from the urine of rats receiving pyridine. If the liver and kidney damage which were observed after administration of pyridine were due to a drainage of methyl groups, then the already methylated product should be incapable of producing the lesions.

EXPERIMENTAL. Young male rats were used and the experiments conducted as described in previous reports (8, 9). The diet referred to here as the basal diet has been fully described previously (8), and was the same as that used in the experiments described in the preceding article of this series (9). It was low in casein and choline, but not so low that significant lesions were produced during the experimental period by the diet alone.

Preparation of Methyl Pyridinium Chloride. Methyl pyridinium chloride was prepared by bubbling methyl chloride through pyridine (17). This product was freed of excess reactants by drying *in vacuo* followed by solution in absolute alcohol, and removal of the alcohol by vacuum distillation. The almost white residue was stored in a vacuum desiccator. Platinum and gold salts prepared as described by His (10), yielded typical crystals. The melting point of the chloroplatinate, $C_{12}H_{16}N_2Cl_2PtCl_4$, was 185-9°C. (uncorrected) with decomposition, compared to the reported value of 184-5°C. (17).

Comparison of Effects of Pyridine and Methyl Pyridinium Chloride in Chronic Feeding Experiments. Four groups containing 6 rats each, were fed the following diets, respectively:

1. Basal diet + 0.1% pyridine.
2. Basal diet + 0.164% methyl pyridinium chloride.
3. Basal diet + 0.328% methyl pyridinium pyridinium chloride.
4. Basal diet + 0.1% pyridine + 0.5% *dl*-methionine.

The animals were observed and weighed daily, and the time of death of each animal was recorded. The organs of most of the animals were examined grossly, and representative ones were studied microscopically.

Examination of Urine for Methyl Pyridinium Salts. Urine was collected from groups of adult male rats injected repeatedly with pyridine or fed diets containing pyridine or pyridine citrate, and from groups fed diets containing methyl pyridinium chloride, respectively, and attempts made to isolate methyl pyridinium salts by the two methods employed by His (10).

Comparison of Acute Toxic Effects of Pyridine and Methyl Pyridinium Chloride. In order to compare the toxicities of pyridine and methyl pyridinium chloride on acute administration, the MLD_{50} of each compound was determined by injecting graduated amounts intraperitoneally into mice.

RESULTS. *Effects of Feeding Pyridine.* The animals receiving the diet with pyridine alone added, gradually lost weight and died in from 2 to 4 weeks (figure 1). The fact that the animals survived longer than those of most similar groups on pyridine diets (8, 9) might have been caused by less frequent feeding and less care in preventing loss of pyridine by evaporation. Examination of the livers and kidneys of this group revealed the acute lesions which have been described (8), and some of the livers exhibited well-marked cirrhosis.

Effects of Feeding Methyl Pyridinium Chloride. The animals in the second group, which received 0.164% methyl pyridinium chloride (equivalent to 0.1%
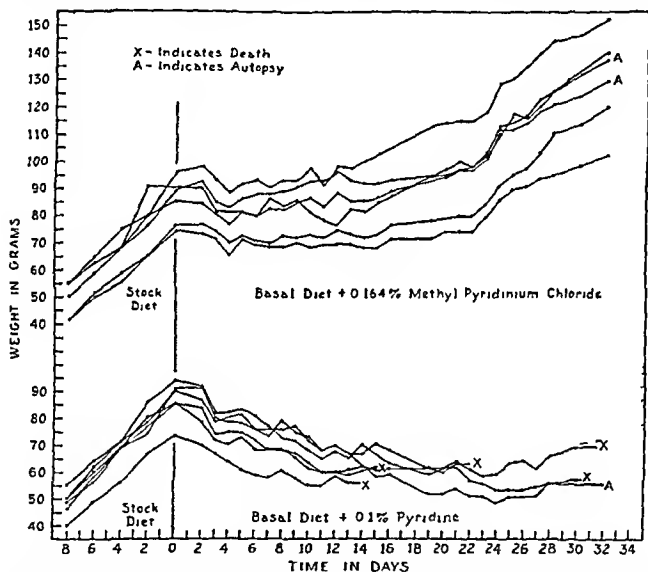
FIG. 1. GROWTH CURVES, COMPARING THE EFFECTS OF DIETS CONTAINING EQUIVALENT CONCENTRATIONS OF PYRIDINE AND METHYL PYRIDINIUM CHLORIDE

pyridine) all survived (figure 1), continued to grow, and were found at autopsy to have essentially normal livers and kidneys.

The results of using 0.328% methyl pyridinium chloride (twice the pyridine equivalent) were similar to those with the lower concentration, except that the growth rate was less rapid (figure 2), perhaps due to the fact that the methylated compound was a central nervous system excitant.

Effects of Feeding Pyridine plus Methionine. The group receiving 0.5% *dl*-methionine in addition to 0.1% pyridine, again demonstrated the protective effect of this supplement (figure 2). The similarity of the growth curves of this group to those of the group receiving equivalent amounts of methyl pyridinium chloride, was striking.

RESULTS OF EXAMINATION OF URINE FOR METHYL PYRIDINIUM SALTS. *After*

Feeding Methyl Pyridinium Chloride.   Considerable amounts of methyl pyridinium hydroxide, isolated and identified as the double platinum salt, were obtained without difficulty from the urine of the rats fed the methylated compound. The substance was apparently eliminated quite rapidly.

After Administration of Pyridine or Pyridine Citrate.   From the urine of rats given pyridine by various methods, a slight precipitate was obtained upon addition of the alkaloid reagents, and in the early experiments it was thought that a few characteristic crystals were formed upon addition of platinic chloride.   How-
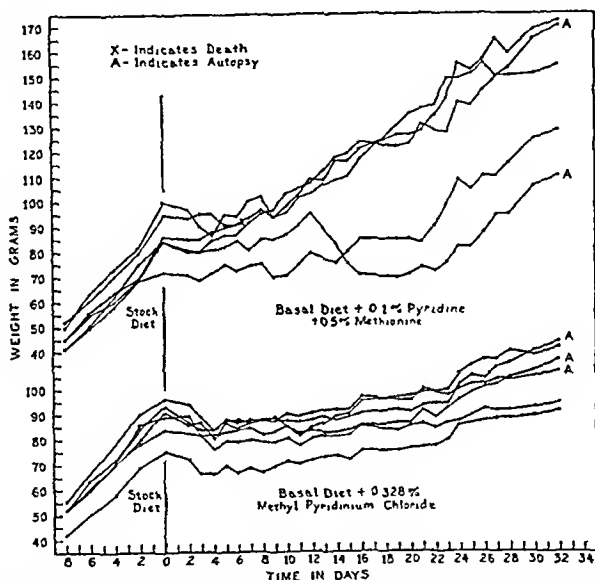
FIG. 2. GROWTH CURVES, SHOWING THE EFFECTS OF TWICE THE EQUIVALENT
CONCENTRATION OF METHYL PYRIDINIUM CHLORIDE, AND OF
PYRIDINE PLUS METHIONINE

ever, these could never be obtained in pure form, and now, after a number of attempts at isolation, it seems fairly certain that the methyl pyridinium salts were never present in significant quantities.   The results were the same when pyridine was administered along with methionine, choline or salt mixture containing an excess of acids.   It should be mentioned that one attempt to obtain methyl pyridinium hydroxide from the urine of a dog fed pyridine, did not prove successful.

ACUTE TOXIC EFFECTS OF PYRIDINE AND METHYL PYRIDINIUM CHLORIDE.   When graduated amounts of pyridine and methyl pyridinium chloride were injected intraperitoneally into mice, it was noted that the MLD of the methylated compound seemed to vary considerably with the volume of the solution in which the injected dose was dissolved, suggesting that the substance was rapidly eliminated

or inactivated, so that the concentration reached in the body was materially affected by the rate of absorption, which in turn was influenced by the concentration of the solution. The effects of pyridine were not greatly influenced by the volume of the solution injected, within the range used, suggesting perhaps that pyridine was not so rapidly eliminated as the methylated compound. When the MLD_{50} of the methylated compound was contained in about 0.5 cc. of solution, the results were as follows:

Pyridine MLD_{50} ..   ..   .   1.2 mgm./gram of body weight.

Methyl pyridinium chloride MLD_{50}... 0.22 mgm./gram of body weight.

Pyridine produced depression of the nervous system, with death frequently occurring after periods of anesthesia of varying length. Methyl pyridinium chloride, on the other hand, was found to be a central nervous system stimulant, causing death following convulsions.

Under the conditions of the experiment, the methylated compound was about eight times as toxic as pyridine, on a stoichiometric basis, despite the fact that the chronic toxicity of pyridine on oral administration to rats was much greater than that of methyl pyridinium chloride. It was intended to repeat these acute experiments on rats, but after publication of the results of Brazda and Coulson (18), which were obtained with subcutaneous injections in rats and which agreed fairly closely with our results in mice, this was not done.

DISCUSSION. Since the work of du Vigneaud and associates (19, 20), which demonstrated that choline and methionine are important sources of labile methyl groups for biological methylations, other observers have suggested that deficiencies of lipotropic substances, with resulting tissue damage, might be produced by feeding certain agents, such as guanidoacetic acid (21) or nicotinamide (22), which are methylated in the body. These observations suggested that a deficiency of labile methyl groups might be a common factor in the production of liver and kidney injury by dietary means and in their production by at least some chemical agents.

While a number of investigators have found that methionine (and also cystine) protected against liver and kidney damage produced by certain toxic agents (23, 24, 25, 26), choline has usually not been highly effective. There has been the observation that the combination of cystine and choline was more efficacious than cystine alone (24). Some have attributed the protective action of the sulfur-containing amino acids against substances producing liver injury, to the potential sulfhydryl groups of these amino acids (23, 26). Other sulfhydryl compounds have been found to afford similar protection under certain circumstances (26), but such protective ability has not always been determined by the presence or absence of sulfhydryl groups (27). A number of enzymes functioning in important biological systems are known to be inactivated by reagents which inactivate —SH groups (28), and many investigators have suggested or demonstrated inhibition of enzyme systems by toxic substances and prevention of this inhibition by certain substances containing —SH groups (29, 30). The relationship of these observations to the production of liver and kidney injury by toxic substances, however, remains to be fully established. The fact that all hepatoxic substances do not produce injury through the same mechanisms, is

emphasized by the protective action of certain hepatoxic substances against others (31, 32).

The observation in the present study that diets containing pyridine, but not those containing the already methylated product of pyridine, produced liver and kidney injury, was considered compatible with the mechanism of pyridine toxicity which was postulated at the beginning, consisting of the production of a methyl deficiency. However, the failure to find methyl pyridinium salts in the urine of rats receiving pyridine, together with the fact that it was easily recovered in considerable quantities after feeding the already methylated product, was considered strong evidence against a drainage of labile methyl groups as the mechanism of the pyridine injury in the rat. This evidence is supported by the previously reported preliminary observations of the ineffectiveness of choline in preventing injury by pyridine (though it was conceivable that the methyl groups of choline might be less readily utilized than those of methionine, in the methylation of pyridine), and of the effectiveness of cystine (7). These results further made it appear unlikely that the protection afforded by methionine against pyridine injury was due principally to the labile methyl group of the methionine molecule, but rather to the methionine molecule as a whole or more likely to the sulfur-containing, cystine-like part of the molecule. The fact that the liver lesions produced by pyridine on diets high in casein and choline, exhibited little visable fat, and that the cirrhosis, characterized by diffuse fibrosis, which was observed under such circumstances, might have occurred entirely as a result of necrosis, have been discussed previously (8). It hardly seems necessary to add that supplements of choline might be expected to produce some modification of the lesions which occur with diets deficient or relatively deficient in choline from the start. The interrelation of choline, methionine and cystine, in-so-far as protection against hepatic and renal damage is concerned, and the question of the relationship of hepatic necrosis and cirrhosis, will be considered in greater detail in a later report.

It seemed probable that the greater chronic toxicity of pyridine evidenced in the form of liver and kidney injury, but greater acute toxicity of the methylated product through entirely different mechanisms, was due to a fundamental difference in the biological action of the two compounds. Another possible explanation of the lesser chronic hepatoxic action of the methylated compound was a more rapid elimination of this substance, which was suggested by several observations but not actually determined, resulting in much less cumulative effect than with pyridine.

SUMMARY

The possible relationship between the mechanisms of the liver and kidney injury produced by certain toxic substances, and the mechanisms of similar injury produced by feeding diets deficient in choline and methionine, was discussed.

The already methylated product of pyridine, methyl pyridinium chloride, when fed in the diet to rats, did not produce liver and kidney injury such as was observed with equivalent concentrations of pyridine.

When methyl pyridinium chloride was fed to rats, it was easily recovered from the urine, but when pyridine was administered, the methylated product was not found in the urine. This observation, together with the previously reported ineffectiveness of choline, but effectiveness of cystine, in affording protection against pyridine injury, was considered substantial evidence against a drainage of methyl groups as the mechanism of the liver and kidney damage produced by pyridine in the rat, and also against the content of labile methyl groups as the explanation of the protective effect of methionine under these circumstances.

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STUDIES ON THE FATE OF NICOTINE IN THE BODY

V. Observations on Relation of Nicotine Dosage to Per Cent Excreted in Urine, Rate of Excretion and Rate of Detoxication

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That administered nicotine appears in the urine has been known for a long time. Thus, Langley and Dickinson in 1890 (1), on the basis of animal experiments, stated that following its injection nicotine is readily found in the urine. Further proof of this has been supplied by Fretwurst and Hertz (2), who isolated nicotine as the dipicrate from the urine of a gardener poisoned by a nicotine insecticide spray, and by Helmer, Kohlstaedt and Page (3), who isolated nicotine as the oxalate and picrate from the urine of persons who smoke.

Urinary elimination begins promptly as demonstrated by Okamoto's (4) observations on dogs and rabbits in which he describes the appearance of nicotine in the urine 10 minutes after administration. Okamoto's observations on animals, as well as those of Noether (5) on man, indicate that, except for trace quantities, persistence of urinary elimination following complete absorption probably does not exceed 10–12 hours. From studies on the toad, Ozawa (6) concluded that nicotine is eliminated by both glomeruli and tubules.

Quantitative studies on the amount of administered nicotine eliminated unchanged have been reported by the following investigators: Bodnar, Nagy and Dickmann's (7) data on smokers who inhale, indicate that about 4 per cent of retained nicotine is excreted in the urine. Corcoran, Helmer and Page (8) found an average nicotine excretion of 0.254 mg. per cigarette smoked. Assuming an average nicotine retention of 3 mg. (9) per cigarette, this would indicate an excretion of about 8 per cent. Corcoran et al. also found a 4.5–12.8 per cent excretion in dogs following administration of 2–3 mg. doses. Haag and Larson (9), in studies on man, found that urinary excretion of nicotine varied with the pH of the urine. At an average urinary pH of 7.1–7.6, 2.3–3.7 per cent of nicotine calculated to be retained from smoke was excreted in the urine, whereas at pH of 4.9–5.7, 9.8–13.0 per cent was excreted. This difference presumably represents decreased reabsorption of nicotine from the urinary tract with increasing urinary acidity. In further studies by these authors on dogs (10), dose levels of 3 mg. per Kg. nicotine led to a urinary excretion of about 10 per cent of this amount. The data of Perlman, Dannenberg and Sokoloff (11) is limited as regards urinary excretion to the finding that there is a definite correlation between number of cigarettes smoked and the concentration of nicotine in the urine.

One can conclude from the above that only a small portion of administered

nicotine is excreted in the urine. However, the dose range is insufficient and the experimental conditions are too inconsistent to be informative as to whether a fixed percentage of the dose is excreted unchanged or whether excretion varies with the dose level. To ascertain this, and, as a corollary, to determine rate of detoxication within the animal body, the following experiments were performed.

EXPERIMENTAL. Female dogs under Dial anesthesia were used. Nicotine in predetermined amount was injected over an 8 hour period via a femoral vein by means of a motor driven syringe which filled and delivered a 1 ml. volume of nicotine solution in isotonic saline once per minute. Nicotine dose levels used were: 0, 3, 6, 12, 15, 24, and 48 mg. per Kg. body weight. Carotid blood pressure tracings were made in the usual manner and respiration was recorded by means of a pneumograph.

With nicotine dose levels that produced respiratory failure during the course of the 8 hour period (15 mg. per Kg. and above), positive pressure artificial respiration was instituted through a tracheal cannula at the onset of failure. Onset was judged by the character of the respiratory tracing and the relatively sharp asphyxial blood pressure rise that accompanies respiratory failure. Once instituted, artificial respiration was maintained until the earliest time following cessation of the 8 hour nicotine administration when the animal could again maintain voluntary respiration. This was judged by testing the ability of the animal to breathe by temporarily interrupting artificial respiration at 15-30 minute intervals following cessation of nicotine injection.

In our early experiments, restoration of respiration, once paralysis had occurred, proved to be the exception rather than the rule, blood pressure gradually falling to shock levels in all cases of failure to restore respiration. Among our efforts to elucidate the cause of this, venous pressure measurements from the region of the right heart were made by means of a catheter passed through the right jugular vein, the nicotine infusion in these cases being made through the same catheter. To prevent coagulation in the catheter, heparin was added to the nicotine injection fluid. With the institution of heparin, restoration of respiration became the rule rather than the exception Therefore, in all subsequent experiments at paralytic dose levels in which nicotine was infused into the femoral vein, heparin (one unit per Kg. per cc. of injection fluid) was added. We have not been able to prove that heparin acted in its usual rôle as an anticoagulant in accomplishing this effect, nor have we any evidence that it influenced nicotine detoxication rate.

In animals in which respiratory paralysis occurred, the reservoir supplying the injection apparatus was changed from nicotine in saline or nicotine in saline plus heparin to saline or saline plus heparin at the end of the 8 hour nicotine injection period and injection continued until return of voluntary respiration.

A retention catheter was inserted into the bladder at the start of the experiment and urine was collected and divided into that formed during 0-24 hours and 24-30 hours following the beginning of nicotine injection. In those cases where respiratory paralysis ensued, the 0-24 hour collection was subdivided into three fractions, namely: that formed prior to respiratory paralysis, that formed during respiratory paralysis, and that formed in the remainder of the 24 hour period.

All urine samples were alkalinized with sodium hydroxide and ether-extracted for 48 hours in a liquid-liquid extraction apparatus, the ether reservoir of which also contained 10 ml. of 1N HCL. The ether extracts were evaporated, the residue made up to 100 ml. volume, and the nicotine content determined by a silicotungstic acid method (12).

RESULTS. The nicotine excretion results at all dose levels are summarized in Table 1 and figure 1. It is apparent that a fixed percentage of the dose is not excreted in the urine at all dose levels, but rather, that with increasing dose an increasing per cent is excreted in the urine. Furthermore, the 24-30 hour

TABLE 1

Effect of dosage on per cent of administered nicotine excreted in the urine

| NO. OF DOGS | DOSE I.V. | AVERAGE NICOTINE EXCRETION (mg./Kg.) | | | | |
		0-24 hr.	24-30 hr.	Total	Total-Blank	% of Dose
	mg./Kg.					
3	0	0.08	0.06	0.14	—	—
3	3	0.27	0.07	0.34	0.20	6.7
4	6	0.57	0.06	0.63	0.49	8.2
3	12	1.03	0.05	1.08	0.94	7.8
3	15	2.12	0.08	2.20	2.06	13.7
4	24	4.83	0.12	4.95	4.81	20.0
3	48	14.57	0.16	14.73	14.59	30.4

TABLE 2

Effect of dosage on rates of detoxication and urinary excretion of nicotine (dogs)

| DOSE I.V. | AV. TIME ONSET RESP. PARALYSIS | PERIOD OF RESPIRATORY PARALYSIS | | | | | |
		Average Duration	Av. Amt. Nicotine Injected	Av. Amt. Nicotine Excreted	Injected Minus Excreted	Av. Rate of Detoxication*	Av. Rate of Excretion†
mg./Kg.	Hrs.	Hrs.	mg./Kg.	mg./Kg.	mg./Kg.	mg./Kg./hr.	mg./Kg./hr.
15	4.67	4.78	6.24	0.70	5.54	1.16	0.15
24	1.95	8.87	18.16	3.73	14.43	1.63	0.42
48	1.03	13.96	41.80	13.65	28.15	2.02	0.98

* Column 6 divided by column 3.
† Column 5 divided by column 3.
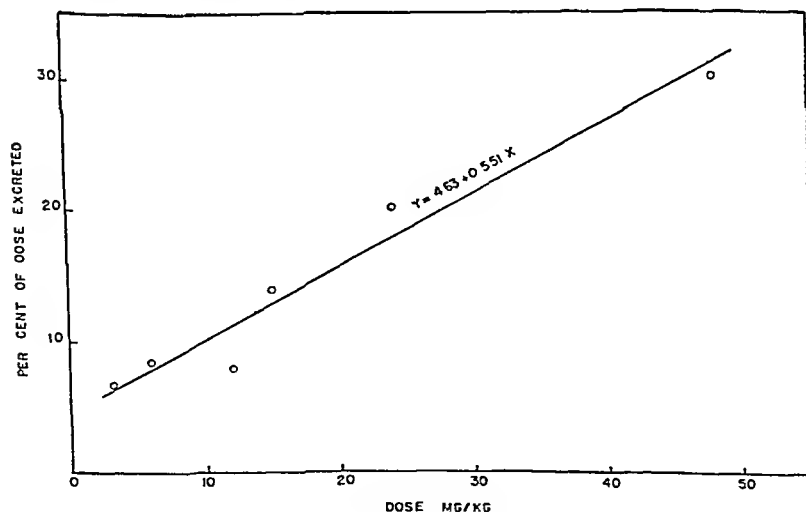
FIG. 1. RELATION OF DOSAGE TO PER CENT EXCRETIONS OF NICOTINE (DOGS)

excretion data show that even with dose levels as high as 48 mg. per Kg., urinary excretion is virtually complete within 16 hours following cessation of administration.
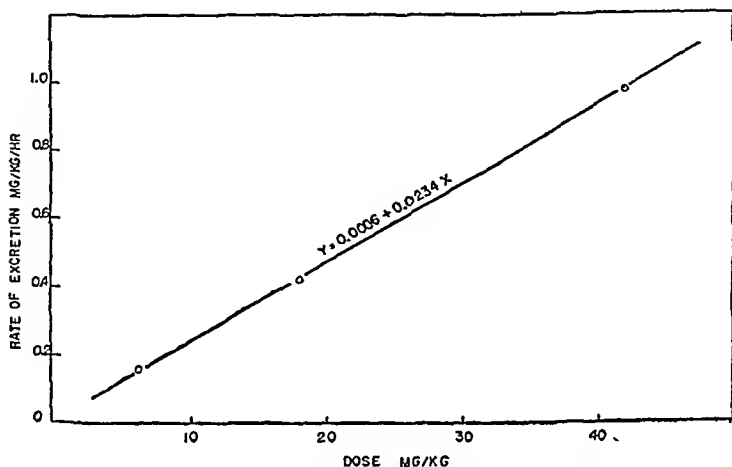
FIG. 2. RATE OF EXCRETION OF NICOTINE (DOGS)
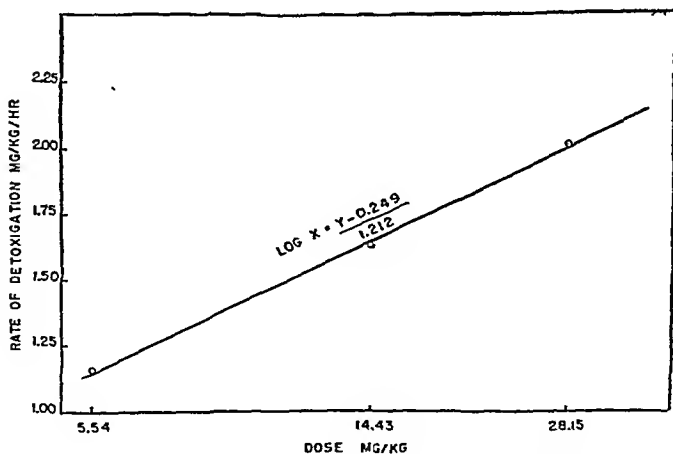
FIG. 3. RATE OF DETOXICATION OF NICOTINE (DOGS)

The nicotine excretion and detoxication rates during the period of respiratory paralysis, for those dose levels that produced paralysis, are summarized in Table 2, and in figures 2 and 3. The calculations in Table 2 are based on the assumption that the body-fluid concentration of nicotine at resumption of voluntary respiration is essentially the same as that at the onset of respiratory paralysis.

The excretion data show that with increasing dosage there is an increased rate of excretion of nicotine in the urine (table 2 and figure 2). Likewise, rate of detoxication increases with increasing dose (table 2 and figure 3). However, whereas rate of excretion bears a linear relation to the magnitude of the dose, rate of detoxication varies with the log of the dose (figures 2 and 3).

CONCLUSIONS

1. Increasing nicotine dosage results in an increasing total per cent excretion in the urine.

2. Increasing dosage results in a linear increase in the rate of urinary excretion of nicotine.

3. Rate of detoxication of nicotine bears a logarithmic relation to dosage.

4. Urinary excretion of nicotine is virtually complete within 16 hours after cessation of administration.

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THE EFFECT OF NIKETHAMIDE ON CORONARY BLOOD FLOW AND CARDIAC OXYGEN METABOLISM*

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Although nikethamide ("coramine") is frequently used clinically in the management of cardiac disease and in the treatment of various cardiovascular emergencies (1), the evidence upon which this use is based consists almost entirely of uncontrolled case reports. A series of preliminary experiments during a student laboratory course in Pharmacology gave no sign of any favorable action by this drug on the mammalian heart with the possible exception of a dilator effect on the coronary arteries. To explore this possibility further, a preparation was devised that would permit a quantitative study of the coronary circulation under conditions closer to normal than had been the case in previous laboratory studies. The method and some of the most important results have already been described (3, 4). The purpose of this paper is to report the findings with regard to nikethamide and also to compare the effects of nikethamide with those of four other drugs commonly used as coronary vasodilators, viz., papaverine hydrochloride, theophylline ethylene diamine, nitroglycerine, and amyl nitrite.

METHODS. These were as described elsewhere (3). The anesthetic was either pentobarbital sodium or morphine-chloralose and the closed-chest spontaneously breathing preparation was used in every case. For injections into the coronary artery, 0.2 cc. volumes were used, all such dilutions being made with warm Locke's solution and kept at a temperature of 37°C. until the moment of injection. Using this method, control injections of 0.2 cc. Locke's solution did not alter coronary flow. The nikethamide used was the 25 per cent solution of Coramine (Ciba) dispensed in glass ampules for intravenous injection. USP or NF preparations of all other drugs were used.

RESULTS. 1. *Nikethamide*. In these studies, nikethamide was used in a varying dosage range (0.125 to 0.750 Grams) which is comparable to the human doses of the drug now advocated (6). The results of these experiments are summarized in figure 1.

Injected into the coronary artery, nikethamide was the least effective of all of the drugs tested. It was only when fairly concentrated solutions (1 to 10 dilution or undiluted) were used that consistent accelerations of coronary blood flow above 50% were noted. Since the pH of nikethamide as taken from the ampule was 6 8, the temporary increase in hydrogen ion concentration may be a contributing factor to this increase in flow with high concentrations of the drug.

There was no consistent change in coronary blood flow when nikethamide was administered intravenously, nor was there any great change in cardiac rate or mean arterial blood pressure. The usual blood pressure response to the intravenous injection of the drug was a sudden fall of 10 to 20 mm. Hg. which returned to its previous level within several minutes.

2. *Other Common "Coronary Dilator" Drugs.* A. Papaverine and Aminophylline—Of the drugs used for comparison with nikethamide, papaverine and aminophylline were the only ones that were consistently associated with an increased coronary blood flow, whether administered intravenously or intra-arterially.

Intra-arterially, papaverine increased coronary blood flow an average of 19 per cent when injected in 0.004 to 0.040 mgm doses, which were the smallest amounts associated with a consistent significant effect. This dosage was not accompanied by changes in mean arterial blood pressure or heart rate as indicated
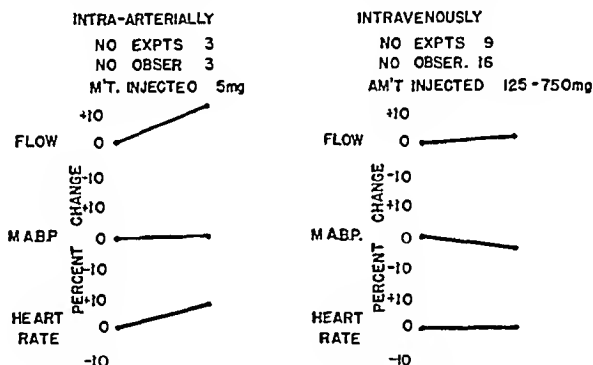
FIG. 1. EFFECT OF NIKETHAMIDE ON CORONARY BLOOD FLOW

in Figure 2 Aminophylline was not very effective until 1.0 mgm amounts were injected intra-arterially and this was associated with a 76 per cent increase in flow occurring simultaneously with a slight increase in mean arterial blood pressure.

The administraton of papaverine intravenously in 15 to 20 mgm amounts was followed by an average increase of 61 per cent in coronary flow. The duration of action was longest with this drug, being 20 minutes on the average in the amounts used. Relatively little change in heart rate or in blood pressure was noted.

The acceleration of flow as noted with intravenous aminophylline was less pronounced (11 per cent) but at the same time a 15 per cent decrease in blood pressure was observed accompanied by a 24 per cent increase in heart rate.

B. Nitroglycerine and Amyl Nitrite—Neither of these two drugs led to consistent changes in coronary blood flow such as might be expected (5). Intra-arterially, nitroglycerine proved to be a potent coronary dilator so long as the

amount injected was not sufficient to produce a systemic effect. Not infrequently, the injection of 0.2 mgm would result in a rapid increase in flow until some of the drug reached the systemic circulation, following which the blood pressure effect would become dominant over the coronary dilator effect, and a decrease in flow would result.

Systemically, nitroglycerine was given by intra-muscular, intravenous, subcutaneous and sublingual routes and in none was the response consistent. In an endeavor to overcome the depressant effects of pentobarbital anesthesia on circulatory reflexes, five experiments were performed using morphine-chloralose
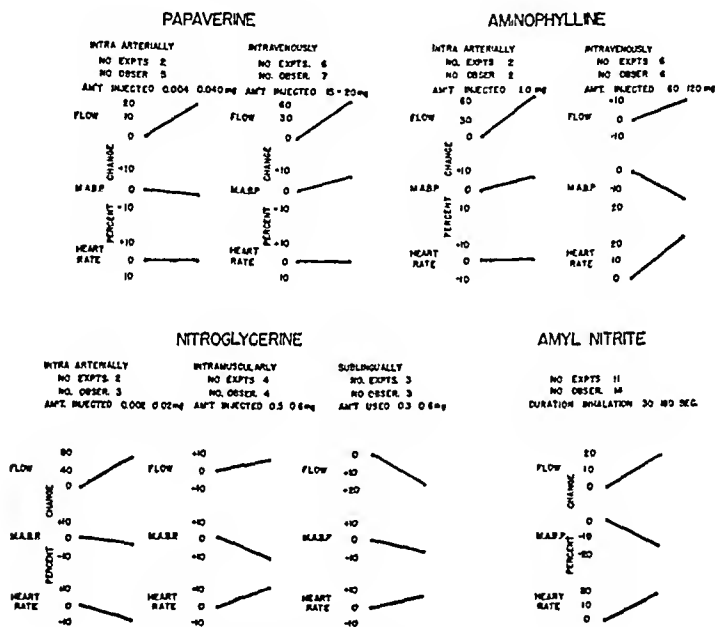
FIG. 2. THE EFFECT OF FOUR COMMONLY USED "CORONARY DILATOR"
DRUGS ON CORONARY BLOOD FLOW

anesthesia but a consistent response was still lacking. If the blood pressure fell, coronary flow rarely increased but if the blood pressure remained fairly constant, a slight increase in flow was usually observed.

The results with amyl nitrite were somewhat better as concerns coronary dilatation, but still were not consistent. Seven of eleven experiments were performed using morphine-chloralose anesthesia. The drug was given by breaking a "pearl" of amyl nitrite in a gauze sponge and holding the sponge within a few centimeters of the tracheal cannula. In several instances, striking increases in coronary flow were noted in spite of considerable decreases in blood pressure (e.g.—in expt. 27, coronary blood flow increased from 38 to 55 cc./100 gm./min.,

while a decrease of blood pressure occurred from 141 to 118 mm. Hg.). Heart rate was invariably increased with this drug. Such occasional findings make the averages for the coronary flow in the amyl nitrite experiments, as indicated in figure 2, appear more impressive than would a study of the data of each experiment.

Thus a summary of the coronary dilator effects of nikethamide as compared with four other commonly used "coronary dilator" drugs reveals that it was the weakest of the five drugs tested when injected intra-arterially and that systemically it had coronary dilator action inferior to papaverine and animophylline and did not even show the occasional definite dilator effect that was exhibited by nitroglycerine and amyl nitrite. This would not support statements that nikethamide definitely increases coronary flow (2). The failure of nitroglycerine and amyl nitrite to show evidence of a coronary vasodilator action, however, led us to suspect that negative findings of this type are insufficient grounds for concluding that no therapeutically useful effect is present.

The possibility remains that drugs could improve the nutrition of cardiac muscle cells without increasing coronary flow. Conversely, it seemed as if an increase in coronary blood flow might occur only secondarily if a drug had such an effect on the heart as to increase the work of the heart or to decrease its mechanical efficiency (work produced/oxygen consumed). In the latter case the supposedly desirable increase in coronary blood flow would in reality be the result of undesirable changes in cardiac work or efficiency.

To examine these two fundamental questions we conducted experiments to obtain further knowledge of factors controlling the volume of blood flowing through the coronary arteries. This was the compelling problem which has led to our work already published on coronary blood flow and cardiac oxygen metabolism (3, 4).

Under the conditions of these experiments we have found (3, 4) that an increased coronary flow can result from many combinations of the following factors: 1.) Increased heart rate 2.) Increased mean arterial blood pressure 3.) Increased cardiac output 4.) Increased left ventricular work 5.) Decreased left ventricular efficiency 6.) Decreased arterial oxygen content 7.) Decreased blood pH. Also we have noted that more oxygen can be made available to the heart at the same or decreased coronary blood flow if there is a decreased demand (decreased work load) or an increased mechanical efficiency.

With the knowledge of the effect of these various factors we felt that we could once more turn to the study of nikethamide and ascertain its effectiveness on the heart without having to rely solely on its coronary dilator effect or to compare such effect with other drugs.

The method used for these final experiments was the same as described in our previous work (4). Coronary blood flow was measured in the anterior descending branch of the coronary artery and venous blood was obtained from a cannula placed in the great cardiac vein or one of its major branches. Oxygen and carbon dioxide content of the blood was determined by the method of Van Slyke and Neill. Cardiac output was obtained by utilization of the direct Fick prin-

ciple with the animal breathing 100 per cent oxygen. Mixed venous blood was obtained from a catheter placed into the right ventricle through the external jugular vein. Four experiments were made.

In all of the four experiments, 1.0 to 1.25 grams of nikethamide (4.0–5.0 cc.) was injected intravenously in dogs averaging 16.1 Kgm. This was equivalent to an average of 70 mgm per kilo. A larger dose was used in this group purposely because with the smaller amounts used previously there were no apparent hemodynamic effects.

A summary of the observations obtained from these four experiments is given in figure 3. In this group a definite increase in coronary flow was observed, amounting to an average of 45 per cent. At the same time there was a 10 per cent decrease in mean arterial pressure and a slight rise in cardiac rate. Cardiac work was increased minimally since a rise in cardiac output counterbalanced the fall in blood pressure. Left ventricular oxygen consumption was found to increase 13 per cent and mechanical efficiency decreased an average of 10 per cent.
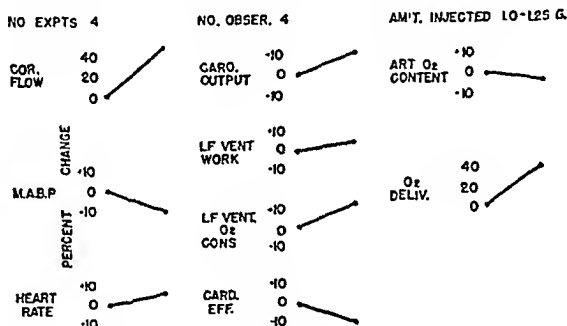
FIG. 3. EFFECT OF NIKETHAMIDE ON CORONARY FLOW AND CARDIAC METABOLISM

In this group an intense respiratory stimulation occurred following the injection of the drug. This response was so intense as to cause us to investigate the effect of a similar dosage of nikethamide in the unanesthetized dog. Six dogs were given the drug intravenously in amounts comparable to that needed in these experiments to increase coronary blood flow (65–80 mgm./kilo). The results are shown in table 1. One dog developed convulsions after the initial injection and the other five showed signs of hyperexcitability, restlessness, increased salivation, and stimulated respiration, sometimes more pronounced in rate and other times in depth. In 3 of 4 dogs in which the drug was repeated in 40 to 90 minutes at the same or decreased dosage level, convulsions occurred and death followed 40 to 120 minutes later.

DISCUSSION. In evaluating the data accumulated in the four experiments summarized in Figure 3, one can divide the observations into two groups such as has been done in table 2.

The hemodynamic factors when considered alone are on the whole quite favorable to nikethamide. There is a considerable increase in coronary flow with

relatively small changes in cardiac output, heart rate, and mean arterial blood pressure. Observations of this type (excluding cardiac output) are of the type that have been reported by groups such as Wegria, et al. (9). One would conclude from such data that nikethamide was a drug of considerable value to increase coronary blood flow.

The other group of factors which we have classified energetic factors, give a much less favorable picture. The increase in oxygen delivered (coronary flow x arterial oxygen content) was somewhat less than the 45 per cent increase in coronary blood flow would indicate. This was because the arterial oxygen con-

TABLE 1

EXP. #	WGT. (Kg)	AMT. INJ.	TIME	RESULT
1	11.3	74/mg./kg.	9:23	Salivation, restless, hyperexcitable
		74/mg /kg.	10:51	Convulsion
			11:30	Dead
2	11.1	80/mg./kg.	9:58	Hyperactive
		80/mg /kg.	10:38	Convulsion
			12:40	Dead
3	8.3	80/mg /kg.	10:08	Restless
4	6.0	80/mg /kg.	10:32	Restless
5	8.7	65/mg./kg.	2:20	Tonic convulsion
		30/mg /kg.	3:17	Clonic convulsion
			5:15	Dead
6	7.0	60/mg /kg.	2:38	Restless
		60/mg./kg.	3:25	Restless, Hyperexcitable

TABLE 2

The cardiac effects of nikethamide

HEMODYNAMIC		ENERGETIC	
Coronary Flow	+45%	Oxygen supplied	+39%
Cardiac Output	+11%	Cardiac Work	+4%
Heart Rate	+5%	Oxygen Consumed	+13%
Mean Art. B.P.	−10%	Ratio $\dfrac{O_2 \text{ Supply}}{O_2 \text{ Demand}}$.	+21%
		Ratio $\dfrac{\text{Work produced}}{O_2 \text{ consumed}}$	−10%

tent decreased, presumably due to the rapid, shallow and thus ineffectual respirations caused by the drug.

Also it was found that the oxygen consumption of the left ventricle increased 13 per cent. Part of this was due to the slight (4 per cent) increase in cardiac work but the more important part was a decrease in mechanical efficiency of the heart muscle, indicating a disproportionate increase in oxygen uptake for the amount of work performed. Thus the analysis revealed that actually only 21 per cent more oxygen was made available to the heart by the 45 per cent increase in coronary flow, and this was primarily at the expense of a reduced mechanical efficiency.

In a previous report (4) we have shown that where cardiac output is increased with blood pressure remaining relatively constant, cardiac efficiency improves. With nikethamide, although the cardiac output increased 11 per cent and blood pressure decreased slightly, actually there was a decrease in efficiency.

Our data on the effects of nikethamide on cardiac efficiency agree very closely with those obtained by Peters and Visscher (8) using a heart-lung preparation. Their average reported decrease in cardiac efficiency was 11 per cent. Certainly our data would support their statement: "This drug cannot be looked upon as a cardiac tonic which it is claimed to be, since in doses that produce any effect at all, there is a loss of cardiac efficiency".

Under the conditions of these experiments, nikethamide was found not to have any significant effect on coronary flow when used in amounts comparable to those used in man (3–5 cc.). When the amounts that would increase coronary flow were injected into unanesthetized animals, convulsions or undesirable symptoms resulted. Presumably one would wish to avoid such effects in patients.

The data accumulated from the unanesthetized animals indicate that the question of toxicity and destruction of nikethamide should be reinvestigated and statements (e.g. 10) about such matters should be more guarded until more information is available. The detoxification figure of 2 mgm./Kgm./minute as reported by Hildebrandt and Mügge for the rabbit (7) apparently does not hold true for dogs and the death of the unanesthetized dogs reported herein indicates that repetition of large amounts of nikethemide is not without danger.

These findings with nikethamide illustrate the point that if one does not investigate 1.) the change produced in the ratio of oxygen supplied to the heart to the oxygen demanded and consumed by the heart (which might be called the nutritional index) and 2.) the effect of the drug on the mechanical efficiency of the heart, one might be lulled into thinking a drug with undesirable effects is a safe and beneficial agent.

SUMMARY

1. In anesthetized spontaneously breathing heparinized dogs with coronary blood flow measured by the bubble flowmeter, the effects of nikethamide, papaverine, aminophylline, amyl nitrite and nitroglycerine on coronary blood flow were measured. In order of their effectiveness these drugs could be listed as follows: papaverine, aminophylline, amyl nitrite, nitroglycerine and nikethamide.

2. An approach necessary for the proper evaluation of the effect of a drug on coronary blood flow is presented. This approach attempts to correlate a) the ratio beween the oxygen supplied to the myocardium and the oxygen demanded by the myocardium (nutritional index) and b) the efficiency of the muscle cells in making the energy transformations necessary to accomplish the work loads imposed on the heart.

3. Utilizing this approach, nikethamide when injected in large amounts was

found to increase coronary blood flow but did so at the expense of a decreased mechanical efficiency.

4. In experiments on six unanesthetized dogs, nikethamide in amounts necessary to produce an increase in coronary blood flow produced convulsions in one dog and undesirable symptoms in the others. Repetition of the same or decreased dose in three animals produced convulsions and delayed death.

5. Our investigations give no indication as to why nikethamide should enjoy such a widespread clinical use in the treatment of cardiovascular disease.

The authors wish to express their appreciation to Dr. Carl F. Schmidt for his suggestions, guidance and interest throughout this problem. They also wish to acknowledge the assistance of Dr. Charles M. Landmesser and Dr. Merel H. Harmel in the performance of some of the experiments.

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BENZOYLCHOLINE AND ATROPINE ESTERASES

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It has been reported that the plasmas of various rabbits differ markedly in their ability to hydrolyze benzoylcholine (1). Indeed, one out of three plasmas possesses negligible activity on this substrate. Similarly, Bernheim and Bernheim (2), Glick and Glaubach (3), and Lévy and Michel (4), as well as earlier workers (see (2)), reported that the blood of some rabbits lacks atropine esterase.[1] In view of these facts it was believed that the hydrolysis of benzoylcholine might be due to the same enzyme that attacks atropine.

The existence of an enzyme which catalyzes the hydrolysis of benzoylcholine, but not that of acetylcholine, was first recognized by Sawyer (5), who tentatively named the enzyme benzoylcholine esterase.[1] Such an enzyme had to be postulated in order to explain the high rates of hydrolysis of benzoylcholine compared to that of acetylcholine found by Sawyer when he assayed these activities of the livers of rabbits and guinea pigs. The only other enzyme that is known to hydrolyze these two esters (pseudo-cholinesterase,[1] Mendel et al. (6)) attacks benzoylcholine one-fourth as rapidly as acetylcholine. Furthermore, when the livers and plasmas of various rabbits were tested for their activity on acetylcholine and on benzoylcholine, no constant relationship between these two activities was found (1). An earlier indication for the presence of the enzyme benzoylcholine esterase is to be found in the data of Nachmansohn and Rothenberg (7). These investigators found that guinea pig kidney hydrolyzes benzoylcholine more rapidly than acetylcholine. Gunter (8) reported that beef kidney contains benzoylcholine esterase and that this enzyme, unlike "pseudo-cholinesterase", is not inhibited by 2×10^{-6} M. eserine   Blaschko et al. (9) have confirmed the presence of benzoylcholine esterase in beef kidney, but have found that the concentration in this tissue is quite low, and that the enzyme from this source is not inhibited by certain compounds which inhibit the benzoylcholine esterase of guinea pig liver.

The present report deals with the somewhat parallel occurrence in the rabbit of enzymes which catalyze the hydrolysis of benzoylcholine and atropine and demonstrates that these activities are due to separable entities. Additional studies are reported on the characteristics of benzoylcholine esterase which differentiate this enzyme from the one that attacks both benzoylcholine and acetylcholine.

[1] The names of enzymes used throughout this paper are those in common usage. In our present state of ignorance of the 'physiological' substrates these names will serve for convenience.

METHODS. The methods used have been reported in a previous paper (1). The substrates used were Benzoylcholine Chloride[2], Atropine Sulfate, Homatropine Sulfate, Acetylcholine Chloride and Acetyl-beta-methylcholine Chloride. Neostigmine Bromide[2] was used as an inhibitor.

RESULTS. *Hydrolysis of Benzoylcholine and Atropine by Rabbit Plasma.* Individual samples of plasma taken from 15 rabbits were tested for their ability to hydrolyze benzoylcholine and atropine. The results are shown in table 1. A certain degree of parallelism between the rates of hydrolysis of benzoylcholine and atropine is evident. However, the ratios of the hydrolysis of benzoylcholine

TABLE 1

Hydrolytic activity of rabbit plasmas on benzoylcholine and atropine

RABBIT NO.	BENZOYLCHOLINE 0.01M	ATROPINE 0.029M	RELATIVE ACTIVITY BENZOYLCHOLINE/ ATROPINE	BENZOYLCHOLINE 0.01M ATROPINE, 0.029M
W	4	0	—	
Y	97	54	1.8	
G	109	68	1.6	
1	0	0	—	
2	15	0	—	3
3	138	119	1.2	121
4	71	45	1.6	49
5	52	31	1.7	38
6	87	58	1.5	63
A1	7	0	—	
A2	7	0	—	
A3	5	0	—	
A4	59	36	1.6	
A5	77	49	1.6	
A6	67	43	1.6	

The activities are given in mm.[3] CO_2 per hour per 0.2 cc. of plasma added to 3 cc. of the stated concentration(s) of the substrate(s).

to that of atropine (table 1, Column 3) are not constant. Some plasmas which did not hydrolyze atropine acted to a slight extent upon benzoylcholine. In those plasmas hydrolyzing both esters the ratio is about 1.6 when both activities are low and approaches unity when the activities are high (rabbit #3). This lack of a constant relationship in the activity of rabbit plasmas on the two substrates was the first indication that both activities were not due to the same enzyme.

Experiments in which an enzyme preparation is allowed to act on a mixture of two substrates are frequently performed to determine whether the individual substrates are being attacked by a single enzyme. In table 1, Column 5 may be seen the results obtained when rabbit plasmas of widely differing activities were allowed to act on mixtures of benzoylcholine and atropine.

[2] A generous sample was obtained from Hoffman-LaRoche, Inc., Nutley, New Jersey, through the courtesy of Dr. Elmer L. Sevringhaus.

The hydrolysis of mixed substrates by the plasmas of rabbits numbered 3 to 6 is only slightly greater than the hydrolysis of atropine alone.   A lack of summation of individual activities of an enzyme preparation on two substrates when both substrates are present in the same mixture is usually interpreted as a competition between the two substrates for a single enzyme.   However, the plasma of Rabbit No. 2 hydrolyzed benzoylcholine and not atropine when a single substrate was present; when both substrates were present, an inhibition of benzoylcholine hydrolysis by atropine was evident.   Inhibition of benzoylcholine hydrolysis by atropine would also account for all the data obtained with mixtures of the two esters.   Experiments in which frog's liver was tested on benzoylcholine and atropine singly and in combinations demonstrate the same phenomenon. The inhibitory action of atropine on benzoylcholinesterase is more dramatic when guinea pig's liver, which has little activity on the former, is tested on mixtures of the two substrates.   This inhibition will be discussed further when the experiments on purified enzymes are described.   Similar experiments with the globulins of rabbit plasma and liver and extracts of guinea pig liver demonstrated an inhibition of benzoylcholine esterase by homatropine when both of these esters were present in the reaction mixture.

Hydrolysis of choline and tropine esters by tissues of rabbits, guinea pigs and frogs.   In order to determine the distribution of benzoylcholine esterase and atropine esterase, a survey was made of certain hydrolytic activities of plasmas, livers and kidneys of rabbits and guinea pigs and of frogs' livers.   These tissues were chosen because most of them have been found to contain hydrolytic enzymes acting upon tropine esters (2, 3).   The substrates tested were benzoylcholine, atropine, homatropine, acetylcholine and acetyl-beta-methylcholine.   The results are shown in Table 2.

The data of table 2 demonstrate the marked differences in the relative activities of the livers of these three species on benzoylcholine, atropine and homatropine. Some rabbit livers possess activities on all three substrates with the order of activity being benzoylcholine > atropine > homatropine.   Some rabbits have little or none of these three activities.   The liver of the guinea pig hydrolyzes benzoylcholine and homatropine rapidly, but has little activity on atropine. The liver of the frog is different from the livers of the previous two species in that it attacks benzoylcholine quite weakly and hydrolyzes both atropine and homatropine.

One finds no constant ratio of benzoylcholine to atropine hydrolysis in these tissues, thus strengthening the evidence for the individuality of the enzymes.

The distribution in rabbit tissues of the hydrolytic activities on the various substrates warrants comment.   There appears to be a fairly constant ratio between the hydrolysis of atropine and of homatropine.   This is not true for benzoylcholine as compared with atropine.   The ratio benzoylcholine/atropine hydrolysis is less in the plasma than in the liver and kidney.   The substrates acetylcholine and acetyl-beta-methylcholine were used in order to show (as previously demonstrated (1)) that the enzyme which attacks acetylcholine in these tissues is related to the specific enzyme found in nervous tissue (and other

tissues), and that the benzoylcholine hydrolysis could *not* be attributed to pseudo-cholinesterase.

In the guinea pig the activity of the plasma on benzoylcholine is due to the enzyme which also hydrolyzes acetylcholine (6). This conclusion is based on the finding of high activity of the plasma on acetylcholine and the low activity on acetyl-beta-methylcholine. Benzoylcholine hydrolysis by guinea pig liver and kidney is due to an enzyme other than that which acts on both acetylcholine and acetyl-beta-methylcholine (5, 7).

TABLE 2

The activities of rabbit, guinea pig and frog tissues on various substrates

ANIMAL	SUBSTRATE CONCENTRATION		BENZOYL-CHOLINE 0.01M	ATROPINE 0.0072M	HOMAT-ROPINE 0.0077M	ACETYL-CHOLINE 0.02M	ACETYL-BETA-METHYLCHOLINE 0.02M
	No.	Tissue					
Rabbit	A1	Plasma	4	0	0	73	57
	3	Plasma	125	97	60	86	52
	6	Plasma	102	66	39	100	53
	A1	Liver	6	1	1	101	93
	3	Liver	91	38	29	120	115
	6	Liver	76	14	9	71	66
	A1	Kidney	4	2	1	10	7
	3	Kidney	27	9	5	13	7
	6	Kidney	16	4	2	12	10
Guinea Pig	1	Plasma	144	0	0	543	42
	2	Plasma	185	0	0	689	42
	3	Plasma	186	0	0		
	1	Liver	107	5	52	20	8
	2	Liver	109	2	45	13	6
	3	Liver	104	2	34	15	5
	1	Kidney	21	2	3	21	6
	2	Kidney	24	1	4	28	7
	3	Kidney	20	1	5	23	8
Frog	1	Liver	1	14	10	28	15
	2	Liver	2	13	12	38	22

Activities as in table 1.

Sexes of animal used: rabbits, female; guinea pigs and frogs, male.

Separation of Benzoylcholine Esterase from Atropine Esterase. The fractionation of the proteins of rabbit plasma and liver by ammonium sulfate precipitation did not separate the activities on benzoylcholine and atropine. Both activities were most concentrated in the pseudoglobulin fraction. Glick et al. (10) found the tropine esterases of rabbit plasma to be in the beta- and gamma-globulins. Since no large changes were made in the ratios of activities on the two substrates, this mode of attack was given up. In some of the following experiments the preparations obtained by precipitation with half-saturated ammonium sulfate were used. These preparations are referred to as plasma "globulin" and liver "globulin."

In a study of the activity of tropine esterase as a function of temperature Glick (11) found that above 40°C. the activity decreased. Since this decrease might be due to the inactivation of the enzyme at moderately high temperatures, the effect of heat on the activities on atropine esterase and benzoylcholine esterase activities was determined. For these experiments rabbit liver globulin which had been dialyzed in the cold against 0.9% sodium chloride was used. The temperature of 48°C. was found to be sufficiently high to destroy the atropine (and homatropine) hydrolytic activity without severely depressing the action on benzoylcholine. Thus, in a typical experiment, incubating a preparation of rabbit liver globulin at 48°C. for 90 minutes reduced the benzoylcholine hydrolysis from 67 to 54 (in mm^3CO_2 per hour per 0.2 cc. of the preparation) and the atropine and homatropine hydrolysis from 19 to 0 and from 13 to 0, respectively.

Thus, heat inactivation of atropine esterase separated sharply the benzoylcholine esterase activity from the former enzyme. The difference in sensitivity to heat was not as great when whole plasma or liver extract was incubated at 48°C.

Drying with acetone does not inactivate benzoylcholine esterase. Thus, one cubic centimeter of a 1:5 liver extract was added dropwise to 10 cc. of acetone, the acetone was poured off the flocculent precipitate and the treatment with 10 cc. of acetone was repeated twice. The solid material was freed of acetone by evaporation under reduced pressure. The residue was taken up in 1 cc. of 0.025 M sodium bicarbonate. This treatment reduced the activity of the liver extract on atropine and acetylcholine without affecting its action on benzoylcholine. The results of these experiments in CO_2 production per hour before and after acetone treatment were as follows: benzoylcholine—59, 58; atropine—14, 4; acetylcholine—58, 8.

Effect of Inhibitors on Benzoylcholine and Atropine Esterases. Some experiments with inhibitors were carried out in order to demonstrate by another method that the hydrolysis of benzoylcholine and atropine by a single preparation were due to different enzymes. Neostigmine proved to be a good inhibitor for demonstrating the differences in sensitivity of rabbit plasma activities on benzoylcholine and atropine. Benzoylcholine hydrolysis is distinctly more sensitive to inhibition by this drug. Neostigmine bromide at a concentration of 10^{-6} M inhibited benzoylcholine hydrolysis 25% but did not affect the hydrolysis of atropine. Benzoylcholine hydrolysis was completely inhibited by 10^{-4} M neostigmine, whereas 10^{-3} M neostigmine was necessary to cause a moderate (30%) inhibition of atropine hydrolysis.

The globulin fractions of both plasma and liver of the rabbit were tested for their activities in the presence of fluoride. Again the benzoylcholine hydrolysis is much more sensitive to this poison than is the hydrolysis of atropine. At 10^{-2} M sodium fluoride the hydrolysis of benzoylcholine by rabbit plasma globulin was inhibited about 50% and by rabbit liver globulin, 75%. At this concentration both of these enzyme preparations when acting on atropine were inhibited to approximately 10%.

A preparation of rabbit liver globulin which had been heated to 48°C. for 90

minutes in order to eliminate the atropine esterase activity was used to demonstrate the inhibitory effect of atropine on the hydrolysis of benzoylcholine. In the presence of 0.01 M benzoylcholine as substrate the inhibitions produced by atropine were as follows: 1.4×10^{-4} M atropine, 9% inhibition; 1.4×10^{-3} M, 54%; 1.4×10^{-2} M, 87%. The total hydrolysis observed in mixtures of benzoylcholine and atropine by rabbit plasma, (cf. table 1) is therefore attributable to the inhibition of benzoylcholine hydrolysis by atropine rather than to a competition of two substrates for a single enzyme. It appears, therefore, that the total acid production in mixture of the two substrates is mainly that resulting from the hydrolysis of atropine with slight additions from the hydrolysis of benzoylcholine.

Benzoylcholine Hydrolysis by Benzoylcholine Esterase and Pseudo-Cholinesterase. Certain properties of the enzyme which hydrolyzes benzoylcholine and not acetylcholine (benzoylcholine esterase) are different from the enzyme (pseudo-cholinesterase) which hydrolyses this substrate as well as acetylcholine. Gunter (8) has reported that there is a marked difference in the sensitivities of the two enzymes to inhibition by eserine.

TABLE 3

Inhibition by neostigmine of two enzymes which hydrolyze benzoylcholine

NEOSTIGMINE	0	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
Rabbit Plasma	121	121	106	43	8	1
Guinea Pig Plasma	192	83	25	14	4	0

2.8 cc. of Neostigmine Bromide of stated concentration in 0 025M NaHCO$_3$ was added to 0.2 cc. of plasma and the mixture allowed to stand at room temperature for one hour before placing vessels in the constant temperature bath. The substrate was 0.2 cc. of 0.15M benzoylcholine chloride dissolved in 0.025 M NaHCO$_3$. Activities as in table 1.

Both rabbit and guinea pig plasmas hydrolyze benzoylcholine, but the activities appear to be due to different enzymes. Further evidence for this is the difference in sensitivity to inhibition by neostigmine, as shown in table 3. The difference does not allow as complete a separation of the two activities as that reported by Gunter, who used eserine as the inhibitor. In the experiments of table 3 the enzyme was in contact with the inhibitor for more than an hour before the substrate was added. The difference in sensitivity is even less marked when the inhibitor and substrate are mixed with the plasma simultaneously. Under these conditions the inhibition of guinea pig plasma is less at 10^{-6} M neostigmine than it is when some time is allowed for 10^{-8} M neostigmine to react with the plasma. These results demonstrate a relatively slow combination of neostigmine with the enzymes.

Benzoylcholine esterase and pseudo-cholinesterase differ greatly in affinity for their mutual substrate, benzoylcholine. In Figures 1A and 1B are shown the results obtained using several substrate concentrations acted upon by the two enzymes. Guinea pig plasma was used as a source of pseudocholinesterase and guinea pig liver for the more specific benzoylcholine hydrolyzing enzyme.

The great affinity of pseudo-cholinesterase for benzoylcholine is shown by the results of experiments with guinea pig plasma. Guinea pig plasma hydrolyzes benzoylcholine at nearly the maximum rate at the lowest practical concentration (0.0003M) and continues its rapid activity until the substrate is spent. Thus, the non-specific enzyme has a substrate concentration at which the activity is half maximum (K_s) much below 0.0003M. The more specific enzyme of the
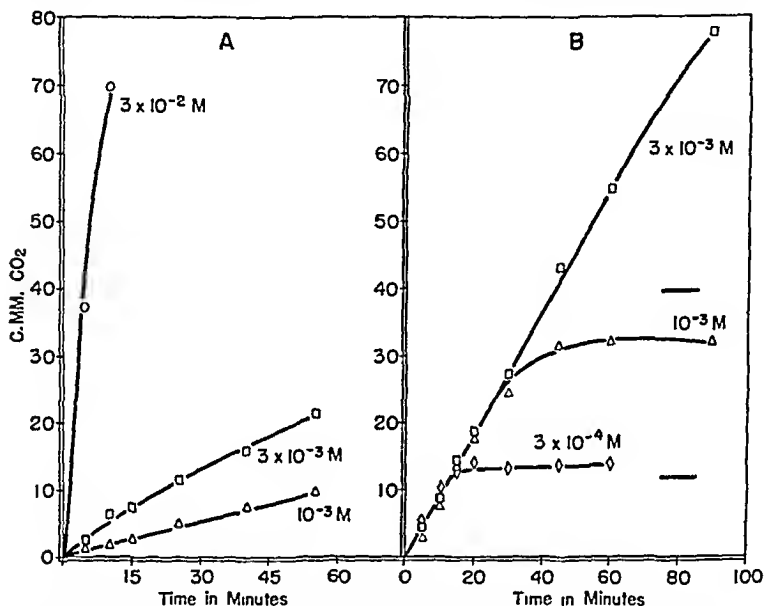
FIG. 1A. HYDROLYSIS OF BENZOYLCHOLINE BY GUINEA PIG LIVER

At zero time 3 cc. of the stated concentration of benzoylcholine chloride in 0.025 M $NaHCO_3$ was mixed with 0.2 cc. of a 1:10 liver homogenate in 0.025 M $NaHCO_3$.

FIG. 1B. HYDROLYSIS OF BENZOYLCHOLINE BY GUINEA PIG PLASMA

At zero time 3 cc. of the stated concentration of benzoylcholine chloride in 0.025 M $NaHCO_3$ was mixed with 0.2 cc. of plasma diluted 1:3 with 0.025 M $NaHCO_3$. In numerous tests of the maximal enzymic or alkaline hydrolysis of the available benzoylcholine chloride preparations only 60% of the theoretical amount of acid production was obtained. The horizontal lines on the graph are the theoretical amounts of CO_2 obtainable for the two lowest concentrations tested calculated on the basis of 60% purity of benzoylcholine.

guinea pig liver shows a much different substrate-activity relationship. Maximum activity is approached at 0.03M benzoylcholine and the K_s is about 0.002. Rabbit plasmas which contain benzoylcholine esterase show the same substrate activity relation as guinea pig liver. Thus, benzoylcholine esterase, a somewhat specific enzyme, has a low affinity for its substrate and pseudo cholinesterase, a less specific enzyme, has a high affinity for benzoylcholine.

DISCUSSION. The evidence presented in this paper supports the previous reports on the existence of an enzyme, benzoylcholine esterase, which is distinct from other known choline ester hydrolyzing enzymes, simple esterases and tropine

esterases. This adds to the list of enzymes with undetermined 'physiological' roles, if such a role exists and the enzymes are not merely essential for detoxication.

Sawin and Glick (12) have reported that the presence or absence of atropine esterase in rabbits is genetically determined. Benzoylcholine esterase, which follows the former enzyme in its distribution in this species, may be similarly determined. However, these authors considered that the enzyme which hydrolyzes monoacetylmorphine was identical with atropine esterase since both are found in parallel concentrations in rabbit plasmas. The genetic basis for the appearance of this enzyme may be indicated by these results, but the statement that the two activities are due to a single enzyme has been questioned by Wright (13).

It appears, therefore, that atropine, morphine and benzoylcholine esterases exist in only a certain per cent of rabbits and that the presence of one indicates that the other two activities are present. The hydrolysis of atropine and of benzoylcholine involve different enzymes. The hydrolysis of monoacetylmorphine may or may not be a function of atropine esterase. This question and the possible identity of morphine esterase and benzoylcholine esterase warrant further study.

Blaschko, Chou, and Wadja (9.14) observed that the benzoylcholine esterase activity of guinea pig's liver is inhibited by certain "atropine like" esters as well as by paludrine and to a lesser degree by the N_5-methyl homologue of paludrine. Our results indicate that benzoylcholinesterase is inhibited by atropine and homatropine. These authors find that 2×10^{-4} eserine does not inhibit guinea pig liver benzoylcholinesterase. Our tests have shown that 10^{-4} M neostigmine and 10^{-4} eserine, when incubated with the enzyme before adding substrate, inhibit the activity of guinea pig liver extracts about 20 per cent and 60 per cent respectively. However, 10^{-4} M neostigmine depresses the benzoylcholinesterase activity of rabbit plasma 100 per cent and the activity of rabbit liver more than 80 per cent, whereas the corresponding inhibitions of these benzoylcholine esterase activities by 10^{-4} M eserine were 70 per cent and 85 per cent. Thus, either differences exist amongst the benzoylcholinesterases from these sources, or, in certain preparations, substances other than the enzyme combine with and thus reduce the effective concentration of the inhibitor.

SUMMARY

1. The plasmas (and also livers and kidneys) of rabbits show great individual variation in their ability to hydrolyze benzoylcholine. This activity may be completely lacking from some plasmas.

2. The activities of the rabbit plasmas on benzoylcholine bear a general, though not quantitative, proportionality to their activities on atropine.

3. The distribution of benzoylcholine esterase and atropine esterase in the tissues of rabbits, guinea pigs and frogs are quite different. Rabbit livers and plasmas have either both activities or neither one; guinea pig livers have only the former; whereas frog livers have essentially only the latter.

4. Benzoylcholine esterase is more sensitive to both neostigmine and fluoride inhibition than is atropine esterase.

5. Incubation of rabbit liver globulin at 48°C. for a sufficient length of time produces a benzoylcholine esterase preparation free of atropine esterase activity.

6. Benzoylcholine esterase and atropine esterase are distinct and separable entities.

7. Benzoylcholine esterase has a low dissociation constant for its substrate (K_s, about 0.002). The dissociation constant for pseudo-cholinesterase and benzoylcholine is much less than 0.0003.

The author wishes to express his thanks to Dr. Frederick Bernheim for valuable advice and criticism concerning this work.

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COMPARATIVE EFFECTS OF ATABRINE AND OF STREPTOMYCIN ON EXPERIMENTAL TUBERCULOSIS IN THE GUINEA PIG

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The suppressive and curative effects of quinacrine hydrochloride (atabrine) on infections with hemoprotozoa and related parasites are well known. It is natural that the usefulness of this drug in bacterial and viral infections should be explored. Goetchius and Lawrence (1) found that certain salts of atabrine had a definite bactericidal effect in vitro on several bacteria such as the pneumococcus and hemolytic streptococci. Campbell and Gilchrist (2) found that *Brucella abortus* was inhibited *in vitro* by 1:5,000 dilution of atabrine but this inhibitory effect was less pronounced in the presence of serum than in its absence. Two patients who had undulant fever were treated with atabrine but the results were not encouraging. Jausion and associates (3) concluded from their experience that atabrine may have some value in the treatment of gonorrhea. Duančić (4) reported favorable results in the treatment of mumps, measles and influenza, and Sylla (5) wrote that atabrine is of aid in the early stages of trench fever.

In 1935 Perrin and Briquel (6) described their efforts to control the fever in twelve cases of pulmonary tuberculosis by giving 0.1 gram of atabrine orally three times daily for five consecutive days. In two cases there was no favorable response; in five the reduction of body temperature was slight but in the remaining five the antithermic effects were pronounced. The patients said they felt better and this effect extended beyond the duration of the reduction in temperature.

Our interest in the possible antituberculosis effects of atabrine has recently been aroused. The purpose of the study reported here was (1) to learn whether the oral administration of atabrine to tuberculous guinea pigs would favorably alter the course of the disease and (2) to compare any therapeutic effect that atabrine may have with that of streptomycin, which has been demonstrated to exert an impressive inhibitory effect on the experimental disease in these animals (7).

METHODS. Twenty-eight normal adult guinea pigs weighing approximately 650 grams each were inoculated subcutaneously over the sternum with 0.1 mgm.[1] of virulent tubercle

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[1] Moist weight. Surface growth from liquid medium is blotted to a dry crumbly mass on sterile filter paper and transferred to a sterile tared watch glass for weighing. The suspension is made by grinding the weighed material with the required amount of sterile physiologic salt solution in a sterile mortar.

bacilli (H37Rv). On the seventeenth day after inoculation the animals were divided into three groups as follows: one group of eight infected guinea pigs was placed on a diet[2] containing 0.1 per cent of atabrine,[3] a second group of eight was treated with 1,500 micrograms of streptomycin[4] four times daily and a third group of twelve animals served as the untreated controls. All animals were weighed twice weekly during the course of the experiment. Fourteen days after treatment was started, blood specimens were obtained from seven of the animals receiving atabrine in order to determine the concentration of the drug in the blood. Treatment with atabrine and streptomycin in the two treated groups was continued for thirty-nine days, at the end of which period all the surviving animals in the treated and control groups were killed. Portions of brain, liver, kidneys, spleen and lungs as well as blood were obtained to determine the concentration of atabrine in these tissues. Tissues were preserved for histologic study and a portion of spleen was removed aseptically for culture.

Atabrine concentrations in the tissues were determined according to the method of Auerbach and Eckert (9) except that 1 cc. of oxalated blood was used instead of 5 cc. as recommended. Control determinations were made on blood from normal guinea pigs and also from the untreated tuberculous animals. Determinations were made on blood and liver to which known amounts of atabrine had been added in order to learn what percentage of atabrine could be recovered from such small amounts as 1 cc. of blood or several grams of tissue.

RESULTS. The concentration of atabrine in the diet was sufficiently great to give it a taste disagreeable to man. However, after a few days the treated guinea pigs consumed as much of their diet as did the untreated animals. Each animal ingested approximately 60 mg. of atabrine per day per kilogram of body weight during the thirty-nine days of treatment. After the first two weeks it was noted that in the animals with a white coat the hair on the feet and belly became yellowish owing to staining with the lemon-colored urine. They all appeared to be as healthy as the controls but there was a gradual loss of weight during the thirty-nine days of treatment, indicating some toxic effects of atabrine at the dosage level used (fig. 1).

Figure 2 presents a comparison of the extent of the tuberculosis seen at necropsy when the surviving animals in the three groups were killed at the end of the experiment. The one untreated animal that died on the fifty-third day of infection was found to have extensive abdominal hemorrhage and widespread tuberculous involvement of the organs of predilection. Four of the eight animals treated with atabrine died shortly before the end of the experiment. One was found to have a rupture of the liver and hemorrhage plus severe tuberculosis in the liver and spleen. Two had sufficient tuberculosis to account for their death but in the fourth there was no demonstrable cause of death. One guinea pig in the group treated with streptomycin died on the eighteenth day of infection after one day of treatment. It had abdominal hemorrhage due to rupture of the liver.

All of the untreated controls had severe tuberculous involvement of the liver,

[2] The basal diet used in this and other experiments of this nature has been described (8).

[3] Furnished through the courtesy of Dr. M. L. Tainter, Winthrop Chemical Company, Inc. Rensselaer, New York.

[4] Furnished through the courtesy of Dr. D. F. Robertson, Merck & Co., Inc., Rahway, New Jersey.
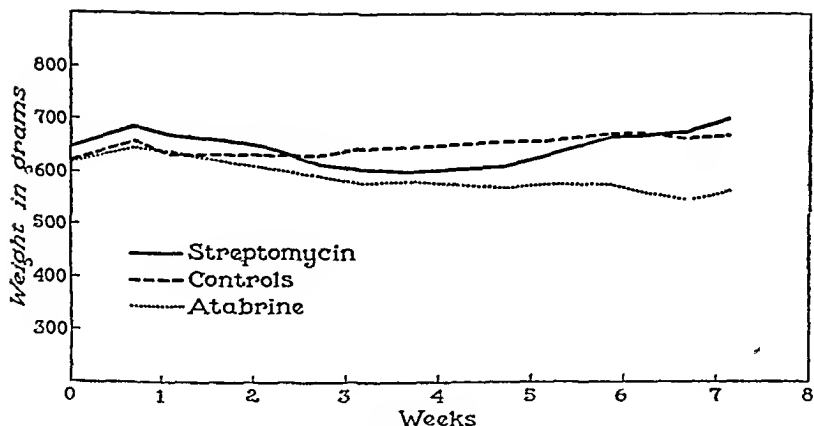
Fig. 1

Comparison of the average weights of the guinea pigs treated with atabrine, those treated with streptomycin and the untreated controls. All animals were weighed twice weekly.
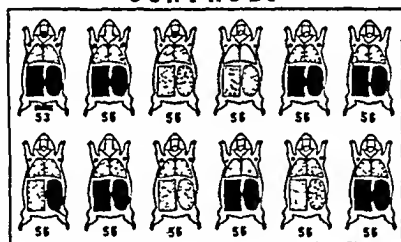
Fig. 2

Schematic representation of the distribution and extent of the tuberculosis seen at necropsy. The number beneath each animal represents the number of days after infection. A black bar indicates that the animal died

spleen and lungs as depicted in figure 2. The animals that received atabrine in the diet had lesions similar in extent and severity to those noted in the controls. In the white and light colored animals of this group the hair of the belly and feet was stained yellow but there was no detectable discoloration of the skin or mucous membranes. The urine in the bladder was lemon colored and the ureters were apparent as fine yellowish lines. The pelvis of each kidney was yellow. In a few guinea pigs the small intestine was yellowish but there was no evidence of enteritis. The liver appeared to be brown instead of the normal color. In the lungs the miliary and nodular tubercles were readily seen as lemon-yellow areas well separated from the normal pink color of the lung. When portions of liver or kidney were placed in 4 per cent solution of formaldehyde the preservative became yellowish within several hours.

The guinea pigs treated with streptomycin had very little gross evidence of tuberculosis as compared to the untreated controls and those treated with ata-

TABLE 1

Comparative effects of atabrine and streptomycin on experimental tuberculosis in guinea pigs
All animals were infected with 0 1 mgm of virulent tubercle bacilli. Treatment continued for thirty-nine days starting on the seventeenth day of infection.

GROUP	INDEX OF INFECTION DETERMINED MICROSCOPICALLY				
	Spleen (Max. 35)	Lungs (Max. 30)	Liver (Max. 25)	Site of inoculation (Max 10)	Average (Max. 100)
1. Controls.....	35.0	21.6	21.6	9.2	87.4
2. Treated with atabrine* .	33.1	22.0	23.1	10.0	88.2
3. Treated with streptomycin†	3.0	3.0	1.8	2.0	9.8

* 0.1 per cent of the diet or approximately 60 mgm. per day per kilogram.

† 1,500 micrograms subcutaneously four times daily.

brine. The comparative extent of the disease in the three groups as determined histologically according to the method described by Feldman (10) is given in table 1. There is no difference between the untreated controls and those given atabrine in the diet, as regards the severity of the disease in the organs of predilection. The microscopic appearance was that of a progressive tuberculous process with no evidence of repair, which was in contrast to the restricted and nonprogressive lesions with fibrosis seen in the tissues from the animals that received streptomycin.

CONCENTRATION OF ATABRINE IN THE BLOOD AND TISSUES. Extracts of blood and tissue prepared by the method of Auerbach and Eckert from normal and eight tuberculous guinea pigs gave very low values of fluorescence. It was possible to recover only 80 per cent of the atabrine added to 1 cc. of blood or to 1 gram of liver, kidney or spleen. Values presented in table 2 for the concentration of the drug in various tissues have been corrected for the small fluorescence exhibited by extracts of normal tissues and for the 20 per cent loss indicated by the recovery experiments.

This study was not designed to learn the effects and disposition of atabrine in the tissues of tuberculous guinea pigs but the few observations in this regard are of interest. It is not known whether the fluorescent material extracted from these specimens represents active atabrine wholly or in part or whether the drug is stored in a modified or detoxicated form. The low concentration of atabrine in the blood as compared to the large amounts stored in the tissues after repeated dosage has been observed by other workers (11–14). Of special interest is the relatively low concentration found in the brain. Apparently the blood-brain barrier is impermeable to atabrine, as it is to certain dyes, toxins and other drugs (15). In spite of the high tissue concentrations of atabrine the only manifestation of toxicity was a slight but continued loss of weight. There was no histologic evidence of any damage to tissue in the gastro-intestinal tract, liver,

TABLE 2

Concentration of atabrine in blood and tissues of tuberculous guinea pigs that received with the diet approximately 60 mgm. of atabrine per kilogram per day

GUINEA PIG	Blood, milligrams per 100 cc.		Tissues, milligrams per 100 gm. after 39 days of treatment				
	14 days of treatment	39 days of treatment	Liver	Kidneys	Spleen	Lungs	Brain
1	0.10	†					
2	0.07*	†					
3	0.17	0.22	87.0	66.0	35.0	72.5	0.31
4	0.08	†					
5	0.11	0.12	17.5	27.5	19.0	21.0	0.09
6	Not done	0.17	59.0	62.5	26.0	50.0	0.21
7	0.07	†					
8	0.08	0.13	25.0	47.5	20.0	36.0	0.20

* Ten days of treatment.
† Not done, animal dead.

spleen or kidneys that could be attributed to the drug but the severe tuberculosis may have masked any such lesions.

If the guinea pig has the ability to modify or detoxicate atabrine in such a manner as to make it inert when stored, this ability may offer an explanation for failure to control the disease in spite of the high concentrations of the drug in the organs of predilection such as the liver, spleen or lungs. Studies *in vitro* show that the growth of the strain of tubercle bacillus used in this study is inhibited by 2.5 mgm. of atabrine per 100 cc. of liquid medium (16), which is much less than the concentrations recorded here for tissues.

The spleens of six of the animals treated with atabrine were cultured. One set became contaminated while five had growth typical of tubercle bacilli. Cultures of the spleens from the untreated animals and from those treated with streptomycin all showed growth.

SUMMARY AND CONCLUSIONS

Eight guinea pigs infected seventeen days previously with virulent tubercle bacilli of the human type were treated for thirty-nine days with atabrine given in the diet at the rate of 60 mgm. per kilogram of body weight per day. A comparison of the extent and severity of the disease in the treated animals with that seen in the untreated control group revealed that the course of the disease was not altered beneficially by atabrine as used. Concentrations of the drug in tissues as determined by a photofluorometric method were found to be many times that required to inhibit growth of the same strain of tubercle bacillus in vitro.

A group of infected animals treated with 1,500 micrograms of streptomycin four times daily for thirty-nine days had lesions with histologic evidence of healing and regression in contrast to the unrestricted, progressive lesions seen in those that received atabrine as well as in the untreated controls.

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TOXICOLOGY OF 1,2-DICHLOROETHANE (Ethylene Dichloride)

IV. Its Detoxication by L-cystine, DL-methionine and Certain Other Sulfur Containing Compounds

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1,2-Dichloroethane, CH_2Cl-CH_2Cl, is a colorless liquid which is widely used as a solvent and for other industrial purposes. Previous reports from this laboratory have dealt with its acute and chronic toxicity for various animals (1, 2), its peculiar ability to render the corneas of dogs cloudy (3), and the influence of dietary factors on its toxicity for young rats. (4).

This paper is concerned with the effect of various compounds in reducing the mortality among young rats given inhalation exposures to dichloroethane. It was found that supplements of DL-methionine and L-cystine were protective when added to a protein deficient-choline deficient diet and also when added to normal diets. The non-sulfur containing amino acids that were tested were ineffective. A number of compounds foreign to the body were found to be detoxifying agents. These either contained an -SH group or could be transformed into sulfhydryl compounds.

A considerable literature has accumulated on the detoxication of aliphatic chlorinated hydrocarbons. A few of the more pertinent references are given in the bibliography (5, 6, 7, 8).

EXPERIMENTAL. Groups of male weanling rats were prepared for several weeks on the different diets. Feeding was *ad libitum* except in one instance (see table 2), and food intake records were kept for nearly every experiment. The rats were then given single or repeated inhalation exposures to 1,000 parts per million (p.p.m.) of dichloroethane. They were observed for at least one week after single exposures and for 3 days after the last of a group of exposures. The operation of the exposure chamber has been described previously (1, 2, 9). Gas samples were analyzed daily as a check on the calculated vapor concentration.

In some of the experiments the chloroform soluble material in aliquot portions of fresh liver was determined by the method of Artom and Fishman (10).

Rats of the Osborne-Mendel strain were used in experiment 1 and in all other experiments animals of the Sprague-Dawley strain were employed. Their average beginning weights in the different experiments varied from 33.3 to 46.1 grams. In 18 of the 21 experiments, the range was 39.4 to 46.1 grams. The several groups within each experiment had average weights that were close together, the deviations seldom exceeding 2 grams. The groups were equal with respect to litter distribution in all experiments except numbers 2, 4, 5, 6, 7, and 8.

The dichloroethane was a commercial product and 8 lots of material were used in this study. Various physical and chemical tests on the first 6 lots are published elsewhere (2). (This reference contains a typographical error in the tabulation of the distillation range. Of a 250 ml. sample, 238 ml. distilled over a range of 0.9°C.) Examination of the last 2 lots was limited to a determination of the index of refraction $N_D^{25°}$, and the values were 1.4442 and 1.4442. The figure quoted in the literature is 1.4443 (11).

Diets. Choline-deficient diets low in casein and high in fat were used in experiments 1 to 10 inclusive. In all other experiments the basal diet contained adequate amounts of choline and either 15 or 25 per cent casein.

The percentage composition of the basal diet for experiments 1 and 2 was: salt mixture,[1] 2; U.S.P. cod liver oil, 3; hydrogenated cottonseed oil,[2] 40; technical casein, 4; dried Brewer's yeast, 4; cane sugar, 47.

In experiments 3 to 10 inclusive the following basal diet was used: hydrogenated cottonseed oil, 38; purified casein,[2] 6; U.S.P. cod liver oil, 5; salts, 2; cane sugar, 49. The diet also contained, in mgm./kg., thiamine hydrochloride, 10; nicotinic acid, 40; pyridoxine hydrochloride, 10; calcium pantothenate, 40; and riboflavin, 20. Once weekly, the rats received 3 mgm. α-tocopherol in 0.03 ml. ethyl laurate orally. For experiments 11 to 21 inclusive, the following changes were made in this diet: (1) hydrogenated cottonseed oil reduced to 6 per cent (2) casein increased to 15 or 25 per cent (3) salts increased to 2.5 per cent (4) addition of 4 mgm. α-naphthoquinone sulfonate, 10γ biotin and 2,000 mgm. choline chloride per kg. of diet. In experiments 12, 13, 15, 17, and 21, the diet also contained 0.5 per cent calcium carbonate and 0.6 per cent $Mg\ SO_4 \cdot 7H_2O$ in addition to that supplied by the salt mixture.

Various sulfur containing compounds were incorporated into these basal diets. The supplements were mixed with the completed diet if the total amounts did not exceed 1.4 grams added to 100 grams of diet. Otherwise, the supplements replaced an equal weight of sugar. The inorganic salts were of C. P. grade. The amino acids and most of the organic sulfur containing compounds were obtained from Merck and Co. or Eastman Kodak Co., and were not further purified. $\beta\beta'$-dithio-propionic acid was synthesized by the method of Stoner and Dougherty (13); its purity was tested by determinations of sulfur content and melting point. S-benzyl-L-cysteine was synthesized by Wood and du Vigneaud's procedure (14) and cysteic acid by the method of Clarke and Inouye (15). Both compounds were analyzed for sulfur and nitrogen and the optical rotation of S-benzyl-L-cysteine was determined to be correct.

RESULTS. In experiments 1 to 10 inclusive (tables 1–4) the basal diet was high in fat, low in protein and deficient in choline. Rats prepared on this diet were quite susceptible to the effects of dichloroethane poisoning. Supplementing the deficient ration with choline had a variable effect on mortality following exposure to dichloroethane. In some experiments partial protection was obtained while in others the mortality was actually increased by dietary additions of choline. Statistical analysis of all of the data in this and the preceding publication (4) showed that choline gave no significant protection. DL-methionine, on the other hand, gave good protection both when mixed into the low casein-high fat diet and when administered as a single oral supplement just before the inhalation exposures (experiments 1 to 8).

L-cystine reduced the mortality when given by stomach tube just before an acute exposure to dichloroethane (experiments 2, 5, 6). It was also effective when mixed in the diet with an equal amount of choline chloride (experiments 5, 6, 7).

When L-cystine alone was added to the choline-deficient basal diet some of the rats developed hemorrhagic kidneys characteristic of severe choline deficiency. This may account for the poor degree of protection shown by L-

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[1] Hubbell, Mendel and Wakeman (12).

[2] CRISCO.

[3] General Brands, Inc.

cystine in experiment 2.   Older rats have been found to be more resistant to the development of hemorrhagic kidneys (16).   For this reason, in experiments 9 and 10 the animals were kept on the basal diet for 19 days before supplementation with L-cystine was begun.   Under these conditions clear cut protection by L-cystine against the effects of dichloroethane could be demonstrated.

TABLE 1

Mortality among rats with different dietary preparation after a single 4-hour inhalation exposure to 1,000 p.p.m of dichloroethane

ADDED TO 100 GRAMS LOW CASEIN-HIGH FAT DIET	ORAL DOSE GIVEN 1 HOUR BE-FORE EXPOSURE	PRE-EXPOSURE PERIOD		NUM-BER OF RATS	MOR-TALI-TY	LIVER FAT CONTENT			
						Lived		Died	
		Av. daily food intake	Av. total weight gain			Mean	S.E.M.*	Mean	S.E.M.
Experiment 1									
gm.	*mgm./kg.*	*gm.*	*gm.*		*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*
		2.9	−4.4	9	70	13.2(3)†		22.0(6)	±2.6
DL-methionine, 1.0		2.7	0.7	9	0	13.7(9)	±0.8		
L-cystine, 0.5		3.0	−0.4	9	20	21.6(7)	±1.5		
Experiment 2									
	water	2.8	3.7	10	100			40.6(10)	±1.5
	L-cystine, 1,000		3.3	10	10				
	DL-methio-nine, 1,000		3.4	11	45			32.7(5)	±2.6
	choline chlor-ide, 1,000		3.4	9	90			39.7(3)	±1.1
DL-methionine, 1.0		2.7	3.6	10	20	9.2(8)	±0.7	5.8(2)	
L-cystine, 0.5		2.5	5.5	10	80	33.7(2)		39.0(8)	±1.8

* Standard error of the mean.

† Figures in parentheses indicate number of rats whose livers were analyzed.

Weanling rats were kept on the choline deficient basal diet for 17 days in experiment 1 and 24 days in experiment 2. Some of the groups had various supplements mixed in the diet from the beginning.   Certain other groups received different compounds in a single dose by stomach tube just before being exposed.   The rats received 1 (one) 4-hour inhalation exposure to 1,000 p.p.m. of dichloroethane.

In experiments 11 to 21 inclusive, (table 5 and fig. 1) male weanling rats were fed choline-adequate diets containing either 15 or 25 per cent casein.   When they had been on the diets for 12 days inhalation exposures to dichloroethane were started.   These were given daily except Saturdays and Sundays.   Various compounds were added to the basal ration, beginning with the third day before the onset of exposures.   Table 5 and figure 1 show that L-cystine, L-cysteine

TABLE 2

Influence of dietary factors on mortality among rats receiving a 4-hour inhalation exposure to 1,000 p.p.m of dichlorethane

ADDED TO 100 GRAMS LOW CASEIN-HIGH FAT DIET	ORAL DOSE GIVEN 1 HOUR BEFORE EXPOSURE	PRE-EXPOSURE PERIOD		NUM-BER OF RATS	MOR-TALI-TY	LIVER FAT CONTENT			
		Av. daily food intake	Av. total weight gain			Lived		Died	
						Mean	S.E.M.	Mean	S.E.M.
Experiment 3									
gm.	mgm./kgm.	gm.	gm.		Per cent	Per cent	Per cent	Per cent	Per cent
		2.6	−3.2	18	67	39.4(6)	±1.5	38.3(12)	±1.1
DL-methionine, 1.0		2.6	1.0	20	0	10.5(20)	±0.7		
choline chloride, 0.7 and lecithin, 3.0		2.5	−1.0	20	85	6.2(3)		11.0(16)	±0.5
Experiment 4									
			−2.1	8	100				
	L-arginine, 1500		−3.1	10	100				
	DL-valine, 1500		−2.1	10	100				
	β-alanine, 1500		−2.2	10	90				
	DL-methionine, 1500		−4.8	10	50				
Experiment 5									
	water	2.7	−0.3	10	100			36.7(6)	±1.7
	gum tragacanth, 1%		−1.5	10	80			38.0(5)	±0.7
choline chloride, 0.7		2.7	0.8	10	50	8.1(5)	±0.7	11.1(5)	±1.2
choline chloride, 0.7 and L-cystine 0.7		2.8	4.2	10	0	5.7(9)	±0.2		
	DL-methionine, 1500		0.4	10	10				
	L-cystine, 1500		−1.3	10	0				
control diet*			29.2	10	0	5.0(10)	±0.2		

* This group of rats received 25 per cent casein and 8 per cent cottonseed oil in the diet. They were pair fed against rats on the unsupplemented low casein-high fat diet.

The period of dietary preparation in experiments 3, 4 and 5 was 23, 27 and 21 days, respectively. The general procedure was as described in Table 1. L-cystine was suspended in gum tragacanth solution.

TABLE 3

Mortality among rats with different dietary preparation and given 2 inhalation exposures to 1,000 p.p.m of dichloroethane

ADDED TO 100 GRAMS LOW CASEIN-HIGH FAT DIET	ORAL DOSE GIVEN 1 HOUR BEFORE EXPO- SURE	PRE-EXPOSURE PERIOD		NUM- BER OF RATS	TOTAL MORTAL- ITY AFTER:		LIVER FAT CONTENT	
		Av. daily food intake	Av. total weight gain		First ex- posure	Second ex- posure	Mean	S.E.M.
gm.	*mgm./kgm.*	*gm.*	*gm.*		*Per cent*	*Per cent*	*Per cent*	*Per cent*
				Experiment 6				
	water	3.1	1.9	10	30	90	35.9(5)	±1.7
	gum tragacanth		2.1	10	30	80	37.6(8)	±1.0
	glycine, 1500		0.5	12	90			
	DL-methionine, 1500		0.2	10	0	0		
	L-cystine, 1500		0.7	10	0	20		
choline chloride, 0.7		3.1	3.2	10	60	100	10.1(9)	±0.7
choline chloride, 0.7 and L-cystine 0.7		3.0	6.8	10	0	60	6.3(9)	±0.5
L-tyrosine, 0.7, glycine, 0.7, DL-tryptophane, 0.7 and choline chloride, 0.7		2.5	1.9	10	50	100	9.4(9)	±0.9
				Experiment 7				
		2.9	1.2	10	90	100	38.5(10)	±1.0
choline chloride, 0.7		3.1	3.0	10	30	50	7.0(8)	±0.9
choline chloride 0.7 and L-tyro- sine 0.7		2.7	0.9	10	50	70	10.3(10)	±1.1
DL-methionine, 1.0		3.1	3.5	10	0	10	8.2(6)	±1.1
choline chloride, 0.7 and L-cystine, 0.7		3.2	6.1	10	0	0	5.7(6)	±0.3
				Experiment 8				
	water		0.5	10	30	80		
	DL-methionine, 1500		−0.2	10	0	10		

The general procedure was as described in Table 1. The period of dietary preparation in experiments 6, 7 and 8 was 21, 24 and 28 days, respectively. Then the rats received a 4-hour inhalation exposure to 1,000 p.p.m. dichloroethane. The second exposure, given 6–7 days later, lasted 7 hours in experiment 6, 5½ hours in experiment 7 and 7 hours in experiment 8.

hydrochloride, DL-methionine, thiourea, thiouracil, $\beta\beta'$-dithiodipropionic acid, and 2-thiobarbituric acid were protective. Thiolactic acid was ineffective by mouth but gave some protection when injected intraperitoneally. Cysteic acid, taurine, S-benzyl-L-cysteine and the inorganic sulfur containing compounds did not protect.

Table 6 shows part of the data on food intake and weight gains. It is evident that some of the compounds protected against death from dichloroethane poisoning, but had no significant favorable effect on food consumption or growth.

TABLE 4

Mortality among rats with different dietary preparation and receiving inhalation exposures to 1,000 p.p.m of dichloroethane

ADDED TO 100 GRAMS LOW CASEIN-HIGH FAT DIET	PRE-EXPOSURE PERIOD		NUMBER OF RATS	TOTAL MORTALITY AFTER:	
	Av. daily food intake	Av. total weight gain		First exposure	Last exposure
Experiment 9					
gm.	*gm.*	*gm.*		*per cent*	*per cent*
	2.3	1.0	7	10	100
L-cystine, 0.7	2.4	1.1	7	0	0
DL-phenylalanine, 0.7	2.4	0.2	7	60	100
DL-isoleucine, 0.7	2.3	0.1	7	30	100
DL-aspartic acid, 0.7	2.8	1.1	7	70	90
L-arginine, 0.7	2.3	0.6	7	70	70
Experiment 10					
		-0.7	10	40	100
L-cystine, 0.7		4.2	10	0	20
L-arginine, 0.7		-0.1	10	60	100
DL-isoleucine, 0.7		0.1	10	80	100
L-tyrosine, 0.7		-0.7	10	30	90

All rats were kept on the unsupplemented choline-deficient diet for 19 days. Then the various compounds were added and dietary preparation was continued for 14 more days. After this, five 4-hour inhalation exposures were given in experiment 9 and two 4-hour exposures in experiment 10.

DISCUSSION. These experiments were begun in order to determine whether rats with choline-deficient fatty livers were more susceptible to poisoning by dichloroethane than control rats. The data appear to show that fat content of the liver is less important than the availability of sulfur containing amino acids insofar as resistance to dichloroethane is concerned. Effective protection was obtained with L-cystine even though the rats had very fatty livers.

Protection against dichloroethane could be demonstrated by a number of compounds all of which can furnish sulfhydryl groups. Thus, L-cystine and $\beta\beta'$-dithiodipropionic acid can be reduced to thiol compounds. Thiourea is thought to exist in tautomeric equilibrium with a thiol form (17) and the same is true for thiouracil and 2-thiobarbituric acid. DL-methionine is metabolically

TABLE 5

Mortality among rats receiving different supplements when given repeated exposures to 1,000 p.p.m. of dichloroethane

CASEIN IN BASAL DIET	ADDED TO 100 GRAMS OF BASAL DIET	NUMBER OF RATS	TOTAL NUMBER OF DEATHS AFTER SUCCESSIVE EXPOSURES Exposure number					FINAL MORTALITY
			2	4	5	10	15	
Experiment 11								
Per cent 15	*gm.*	15	8	15				*Per cent* 100
15	L-tyrosine, 0.7 and glycine, 0.7	15	8	11				73
25		15	2	8				53
15	DL-methionine, 1.0	15	0	4				27
Experiment 12								
15		15	3	6	11	12		80
15	thiomalic acid, 0.6	15	1	3	10	12		80
15	thiolactic acid, 0.6	15	4	10	10	15		100
15	L-cystine, 0.6	15	0	0	0	0		0
Experiment 13								
15		15	4	13	14	14		92
15	sodium chloride*	15	1	3	8	14		92
15	sodium chloride†	15	1	7	10	14		92
15	sodium thiolactate*	15	1	2	5	5		33
15	sodium thiolactate†	15	0	7	9	9		60
Experiment 14								
15		15	4	10	13	13	14	92
15	L-cystine, 0.7	15	0	0	0	0	1	7
15	L-cystine, 0.7‡	15	0	0	0	0	0	0
15	thiourea, 0.7	15	0	0	0	0	0	0
15	thiouracil, 0.7	15	0	0	1	1	1	7
Experiment 15								
15		30	7	16	22	27		90
15	L-cystine, 0.3	15	0	2	2	2		13
15	L-cystine, 0.7	15	0	0	2	3		20
15	thiourea, 0.19	15	0	0	0	0		0
15	thiourea, 0.44	15	0	0	1	1		7
Experiment 16								
15		15	6	11	11	11	13	87
15	L-tyrosine, 0.7 and glycine, 0.7	15	4	13	13	14	14	92
15	Na$_2$S$_2$O$_3$·5H$_2$O, 0.7	15¶	6	13	14	14	14	92
15	taurine, 0.7	15	6	9	10	12	13	87
15	dithiodipropionic acid, 0.6	10	1	3	3	4	4	40
15	L-cystine, 0.7	15	0	2	2	2	2	13

TABLE 5—*Concluded*

CASEIN IN BASAL DIET	ADDED TO 100 GRAMS OF BASAL DIET	NUMBER OF RATS	TOTAL NUMBER OF DEATHS AFTER SUCCESSIVE EXPOSURES					FINAL MORTALITY
			Exposure number					
			2	4	5	10	15	
Experiment 17								
per cent	*gm*							*per cent*
15		15	2	6	10	12		80
15	S-benzyl-L-cysteine, 1.2	15	10	13	13	14		92
15	cysteic acid, 1 0	15	3	8	10	12		80
15	L-cysteine ·HCl, 0.39	10¶	1	2	3	3		30
15	2-thiobarbituric acid, 0.7	15	0	0	1	4		27
15	L-cystine, 0 3	15	0	2	2	3		20
Experiment 18								
25		15	0	8	8	9		60
25	1,2-dimercaptopropanol§	15¶	4	7	10	11		73
25	NaCNS, 0 25	15	3	8	8	8		53
25	L-cystine, 0 3	15	0	1	3	4		27
25	L-cystine, 0 5	15	1	3	4	5		33
25	L-cystine, 0.7	15	0	0	0	0		0
Experiment 19								
25		15	6	13	14	14		92
25	Na₂SO₄, 0 82	15¶	5	9	13	13		87
25	dithiodipropionic acid, 0 26	15	5	11	11	11		73
25	dithiodipropionic acid, 0.44	15	1	5	7	7		47
25	ditbiodipropionic acid, 0 61	15	0	6	8	8		53
25	L-cystine, 0 7	15	0	2	3	3		20
Experiment 20								
25		15	6	11	14	14		92
25	Na₂S·9H₂O, 1.0	15	4	14	15	15		100
25	p-aminobenzoic acid, 1.0	15	4	14	15	15		100
25	2-tbiobarbituric acid, 0.7	15	0	7	9	10		67
25	L-cystine, 0 7	15	1	4	4	4		27

* Daily intraperitoneal injections of 0.19 millimoles dissolved in 1.22 ml. of water.

† Daily intraperitoneal injections of 0.28 millimoles dissolved in 1.84 ml. of water.

‡ Supplement given for 12 days preceding onset of exposures. For all other groups in this table supplements were begun on the third day before starting inhalation exposures.

§ Daily intramuscular injections of 1 mgm

¶ Indicates non-littermate groups. All of the other groups in each experiment were equal with respect to litter distribution.

The concentration of dichloroethane was 1,000 p p m. in the atmosphere. Each exposure lasted 4 hours in those experiments where the basal diet contained 15 per cent casein and 7 hours where the diets contained 25 per cent casein. The total number of exposures was 10 or 15 as indicated above. Experiment 9 was an exception for the rats received three 7-hour exposures followed by one 4-hour exposure.

related to cysteine.   Cysteine was not protective when its sulfhydryl group was blocked as in S-benzyl-L-cysteine. The sulfhydryl compounds thiolactic and thiomalic acid were ineffective by mouth, but the former gave some protection when injected intraperitoneally. 1,2-Dimercaptopropanol did not protect against the effects of dichloroethane, possibly because the inherent toxicity of BAL did not permit giving large enough doses.
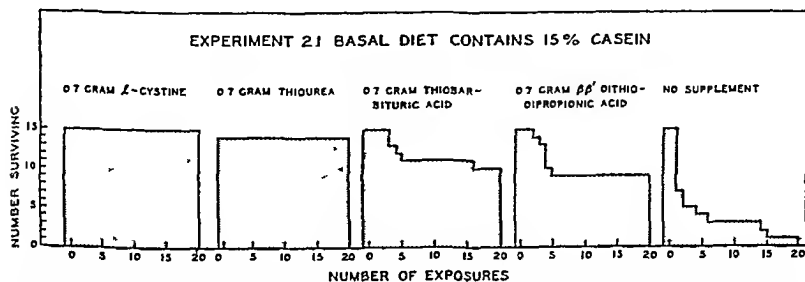
FIG. 1. EXPERIMENT 21. BASAL DIET CONTAINS 15% CASEIN

Mortality among rats receiving different dietary supplements and given repeated exposures to 1,000 p.p.m. of dichloroethane. The animals were exposed 4 hours daily, 5 days a week.

TABLE 6

Average daily food intake and weight changes of young rats during period when exposures to 1,000 p.p.m. dichloroethane were given

EXPERIMENT NUMBER	ADDED TO 100 GRAMS BASAL DIET (15% CASEIN)	PERIOD OF TIME DURING WHICH RATS RECEIVED FIVE 4-HOUR EXPOSURES WEEKLY	
		Av. daily food intake	Av. daily gain in weight
	gm.	*gm.*	*gm.*
17	L-cystine, 0.3	5.4	2.1
17	L-cysteine·HCl, 0.39	5.1	2.3
17	2-thiobarbituric acid, 0.7	4.5	0.7
15	thiourea, 0.19	3.6	0.3
21	thiourea, 0.7	3.6	0.1
21	L-cystine, 0.7	7.3	3.0
21	dithiodipropionic acid, 0.7	4.2	0.2
17, 12, 21, 15		3.9	0.6

Only rats which survived the entire period of exposures were used in this table. In order to have a large enough group of unsupplemented rats, all of the survivors from 4 experiments are placed in 1 group.

Zahl, Drasher and Hutner (18) found that thiouracil had some protective effect against dichloroethane for mice and pointed out its goitrogenic potency. They noted that sulfanilamide and p-aminobenzoic acid were found to be protective against dichloroethane in mice (1) and these also are goitrogens. In the present study the goitrogenic agents thiouracil and thiourea protected rats against dichloroethane but p-aminobenzoic acid was ineffective. 2-Thiobarbi-

turic acid, L-cystine, DL-methionine and L-cysteine hydrochloride, all of which protect against dichloroethane, are not goitrogenic.

SUMMARY

The sulfur containing amino acids L-cystine and DL-methionine protected young rats against death from inhalation exposures to 1,2-dichloroethane. This was demonstrated when these amino acids supplemented low protein-choline deficient diets and also when they were added to normally constituted rations. Supplements of choline chloride did not give any significant protection. Nine sulfur-free amino acids were found to be nonprotective.

Other sulfur containing compounds were tested for their ability to reduce the toxicity of dichloroethane. The inorganic compounds, Na_2SO_4, $NaCNS$, Na_2S and $Na_2S_2O_3$ were ineffective. Cysteic acid, taurine and S-benzyl-L-cysteine did not protect. Protection was obtained with thiourea, thiouracil, 2-thiobarbituric acid, $\beta\beta'$-dithiodipropionic acid and L-cysteine hydrochloride. Thiolactic acid was ineffective by mouth but afforded protection when injected intraperitoneally. It is pointed out that all of the protective compounds can furnish sulfhydryl groups.

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